**De novo mapping of genetic interactions via combinatorial CRISPR-Cas9 screens**

**We develop a CRISPR-Cas9 based approach to systematically map genetic interactions by targeting single and pairs of genes in high throughput, and build a novel analysis method for scoring interactions in competitive growth experiments. Using this methodology we assay a panel of cancer-specific genes across multiple cell lines and unravel networks of conserved and private interactions of therapeutic relevance. This method is broadly applicable for mapping genetic interactions in eukaryotic systems.**

Occasionally, simultaneous mutations in two genes produces a phenotype that is surprising in light of each mutation’s individual effect. This phenomenon, which defines genetic interaction, can reveal functional relationships between genes, complexes and pathways. Assaying genetic interactions in a systematic and large-scale fashion thus has broad applicability for advancing fundamental understanding of biological systems as well as for therapeutic development. For instance, in cancer, a vast range of somatic mutations, copy number aberrations, and epigenetic alterations underlie cellular transformation. Development of precision therapeutics here will consequently entail sifting through the vast number of observed mutations to identify targetable genetic vulnerabilities within each patient1. Furthermore, it is clear from the observed non-random combination of driver events in tumors that genetic interactions have an important role in tumor development and progression2. While many systematic approaches have been employed to study the effects of gene mutations, such as large-scale screens using RNAi, ORFeomes, and CRISPR-Cas9 to study essential genes3-5, these single-gene screens cannot directly identify dependencies and interactions between genes. To identify dependencies, some studies have thus taken alternative approaches: loss-of-function screens coupled with known mutations6, gene expression profiling, shRNA–mediated drug sensitization screens7, and double RNAi8-10. However, each method poses technical challenges that limit their ability to systematically screen for functional genetic interactions.

To enable *de novo* mapping of genetic interactions and networks, we report a CRISPR-Cas9 screening methodology for systematic targeting of single and pairs of genes in high-throughput. In the CRISPR-Cas9 system, a guide-RNA (gRNA), in complex with the Cas9 protein, targets genomic sequences homologous to the gRNA11-13. Targeting new genomic elements thus only requires modifying the gRNA sequence, and by coupling appropriate effector domains one can enable a range of targeted genome editing and regulation capabilities14. CRISPR also enables facile multiplex targeting via delivery of multiple gRNAs per cell15. Here we combine multiplex targeting with array-based oligonucleotide synthesis11,16-19 to create dual-gRNA libraries covering up to 105 defined gene pairs (**Figure 1A**). In these libraries, each construct bears two gRNAs, with each gRNA designed to target either a gene or a scrambled non-targeting sequence absent in the genome. Thus, all combinations of gene-gene (double gene perturbations) and gene-scramble (single gene perturbations) can be exhaustively assayed for effects on cell growth.

Preparation of the dual-gRNA library involves a two-step cloning process whereby each synthesized oligonucleotide is assembled progressively with promoters and 3′ gRNA scaffolds (**Figure 1B**). This multi-step protocol is critical since array-based oligonucleotides from commercial vendors have a maximum length of ~300 bp, while a dual-gRNA cassette is ~1000 bp in size; thus, additional steps of cloning are needed to reconstitute the full sequence. We optimized the library efficacy by eliminating large repeat sequences in the dual-gRNA vectors, as such repeats could potentially compromise both viral production and sequencing quality. Towards this goal we chose non-homologous polymerase III promoters (hU6, mU6) based on their comparable activity15. We also explored mutagenized gRNA scaffold sequences that further increased sequence diversity but maintained the primary hairpin loops in the gRNA scaffold (via utilization of G-C vs. A-U interactions); experiments indicated that while engineered versions 2 and 3 were active, the wild-type scaffold and version 4 showed the most consistent activity (**Supplementary Figures 1 and 2**) and were thus utilized for all subsequent studies. We also confirmed that the two gRNA positions in the construct were equally functional (i.e. hU6-gRNA and mU6-gRNA), and thus the dual-gRNA libraries did not need to include all positional orientations for a gRNA (**Supplementary Figure 3**).

To systematically quantify gene fitness and genetic interactions, we also built a novel analysis method that integrates data across multiple samplings. Specifically, we collected and analyzed cells at four time points: day 3, day 14, day 21 and day 28. We fit the log2 relative abundance of each construct vs. time with a growth curve, the slope of which reflects the fitness of the construct (**Figure 1C and Supplementary Figures 4, 5, 6**; **Methods).** Single-gene fitnesses as well as the genetic interactions are found by robust fitting of the equations , where is the fitness of the double-knockout construct, and are the fitnesses of the individual probes and is the interaction between probes and . Despite optimization of sgRNA probe sequence, the efficacy of knockout generation can be quite variable [REF], so we rank and weight the three probes for each gene according to the absolute value of fitness, (**Supplementary Figure 5**). We calculate the fitness of each gene as the weighted mean of the three sgRNA fitnesses. In the current weighting scheme, only the two fittest probes have non-zero weights.A gene-level score is calculated as the weighted mean of the nine constructs representing the gene pair g, g’. Again, in the current weighting scheme, only the four fittest constructs have non-zero weights. **A negative score indicates slower-than-expected growth, suggesting synthetic sickness or lethality, while a positive score indicates faster-than-expected growth, suggesting epistasis20.

Using the above methodology, we constructed a combinatorial library to exhaustively interrogate the network of genetic interactions among a panel of 73 tumor suppressors genes (TSG) and cancer-relevant drug targets (**Figure 2A, Table S1**). Three gRNAs were designed against each gene (**Methods**); additionally, three gRNAs were designed to be non-targeting controls. Dual gRNA constructs were synthesized for all pairwise gRNA combinations between genes (double knockouts) and between genes and non-targeters (single knockouts). This design resulted in 9 pairwise gRNA constructs for each of the 2,628 double gene knockouts and 73 single gene knockouts, for a total of 24,309 constructs (**Table S2**). Using this design, we conducted a negative selection screen by measuring the relative change in gRNAs over 28 days in a population of cancer cells grown *in vitro* (**Methods**).

Using this pipeline, we assayed genetic interactions in two cancer cell lines, HeLa, a cervical cancer cell line driven by Human Papilloma Virus (HPV), and A549, a KRAS lung cancer cell line driven by KRAS G12S mutation. We found that measurements of single gene fitness between biological replicates in the same cell line were remarkably well correlated; XX, *p* = YY for HeLa and XX, *p* = YY for A549 (**Supplementary Figure 7A,D**). Similarly, the scores for each interaction were well correlated between biological replicates XX, *p* = YY for HeLa and XX, *p* = YY for A549 (**Supplementary Figure 7C,F**). We z-score normalized the scores for each cell line; at a threshold of z < -3 there were 32 synthetic lethal hits in HeLa and 61 in A549, while at z > 3 there were 28 epistatic hits in HeLa and 1 epistatic hit in A549 (**Table S3**). Comparing the two cell lines, we found greater correlation in single gene fitness, r = 0.49, *p* = 1.4 x10-5 (**Figure 2C**), relative to genetic interaction, r = 0.XX, *p* = XX x10-Y (**Figure 2D**). As expected, we found that knockout of drug target genes had a more negative growth impact relative to TSG, *p* = XX for HeLa, *p* = YY for A549 (**Figure 2B, Supplementary Figures 8, 9**).

To identify genetic interactions conserved between the two cell lines, we multiplied the z-scores for HeLa and A549 for each interaction to create a z-product. This conserved network (**Figure 3A, Table S3**) contains many therapeutically relevant interactions, including CHEK1-MAP2K1 and ATM-CDK9, which were validated in micro-titer viability assays using chemical inhibitors of each gene (**Figure 3B,C**). The strong synthetic lethal interaction between CHEK1 and MAP2K1 (also known as MEK1) suggest that inhibitors of CHEK1, of which several are in clinical development21, should be tested in combination with MEK1 inhibitors, such as the FDA approved Trametinib. Additionally, ATM is frequently mutated in both solid and hematologic malignancies22, and its synthetic lethal interaction with CDK9 suggests these ATM mutation tumors should be sensitive to CDK inhibitors such as the FDA approved Palbociclib. Several private interactions for each cell line were also validated, including CHEK1-ADA and CDK9-PRKDC in A549, and CDK9-CASP8 and CHEK1-ATM in HeLa. The three conserved epistatic interactions, CDKN2A-PTEN, TP53-PTEN, and NF1-PTEN, are all enriched for co-occurrence in TCGA pan-cancer dataset, suggesting these pro-growth interactions are common to many cancer types. Of the 91 total unique SL interactions reported here, XX have been previously reported in literature (Table S3).

We note a recent study, CombiGEM, utilizing a multi-gRNA CRISPR-Cas9 approach23. While the studies are complementary, we also note some key differences. First, our methodology utilizes a deterministic dual-gRNA library construction approach whereby each dual-gRNA pair is specified during oligonucleotide synthesis, thus enabling the library size to be fully specified and also enabling arbitrary user-specification of gRNA pairs. Second, we develop a novel computational framework that utilizes a longitudinal analysis integrating data across multiple time points and also systematically ranks gRNAs to minimize variability between replicates and improve the overall screen signal. Consistent with our studies (ref), we also note the importance of gRNA efficacy on the screen signal and anticipate that improvements in design here will be critical to further scale these experiments. Taken together, we anticipate our integrated approach will have broad utility in CRISPR-based genetic interaction screens.

In summary, these combinatorial screens have successfully identified many therapeutically-relevant genetic interactions in cancer. Notably, this experimental and analytical framework is not unique to cancer cell cells, but rather can be readily applied to systematically determine genetic interactions and pathway architecture of complex biological systems3 in any eukaryotic system amenable to lentiviral transduction and growth in culture. Additional, this framework for dual-gRNA libraries is also readily extendable to CRISPR-mediated genome activation and repression screens. Moving forward, our approach paves the way for systematic determination of the network architecture of cancer, with two-fold application: improving our understanding of how networks of genes influence tumorigenesis, and aiding in the development of precision therapeutics via new druggable synthetic lethal interactions.

**Acknowledgments**

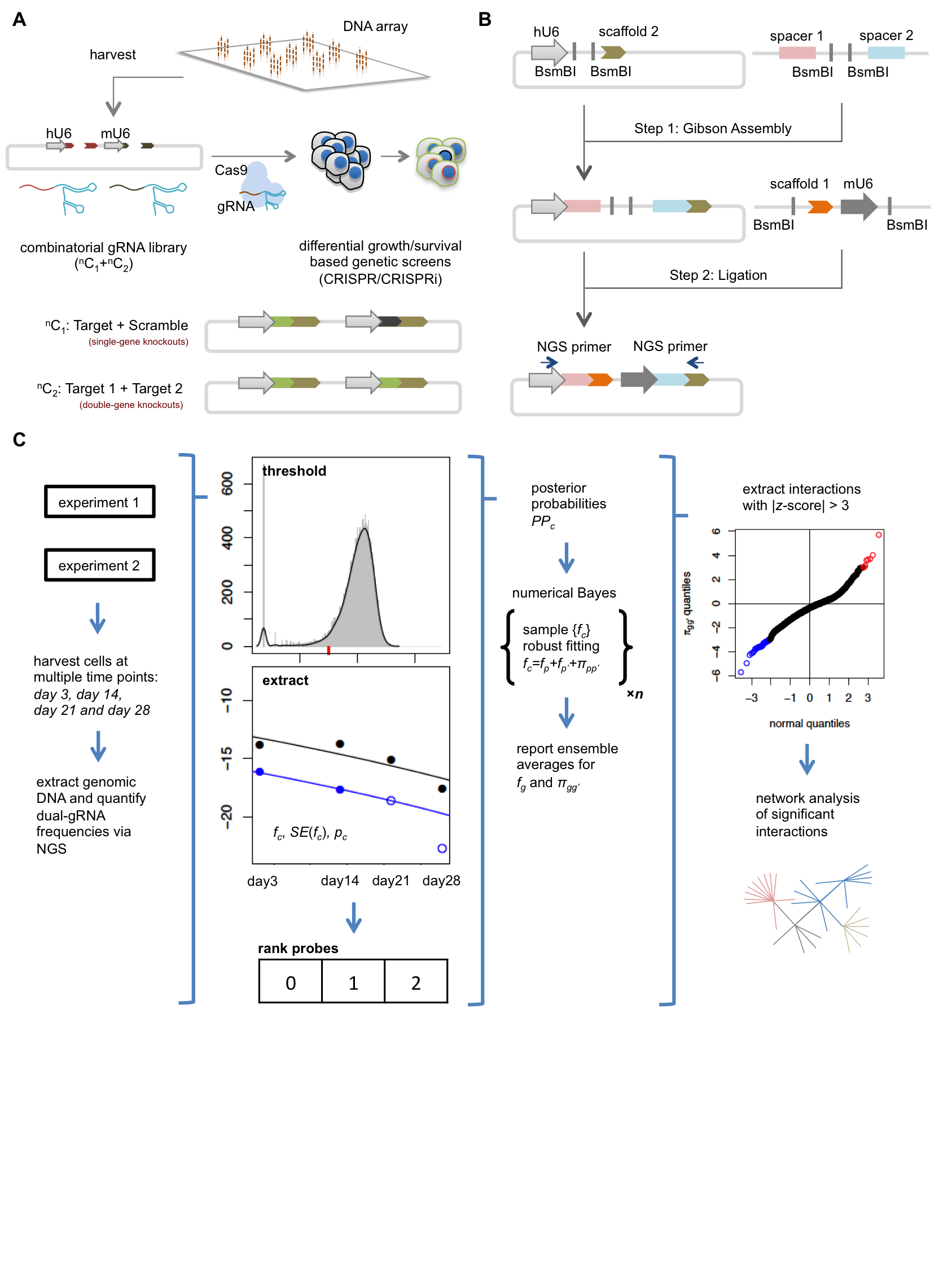
This work was generously supported by UCSD Startup Funds (PM), Burroughs Wellcome Fund (PM), March of Dimes Foundation (PM), Kimmel Foundation (PM), the California Institute of Regenerative Medicine (TI, JPS), and the National Cancer Institute (TI, JPS, CA184427). Work by R. Sasik and A. Birmingham was in part supported by the UCSD Clinical and Translational Research Institute (CTRI, UL1TR001442).

**Figure Captions**

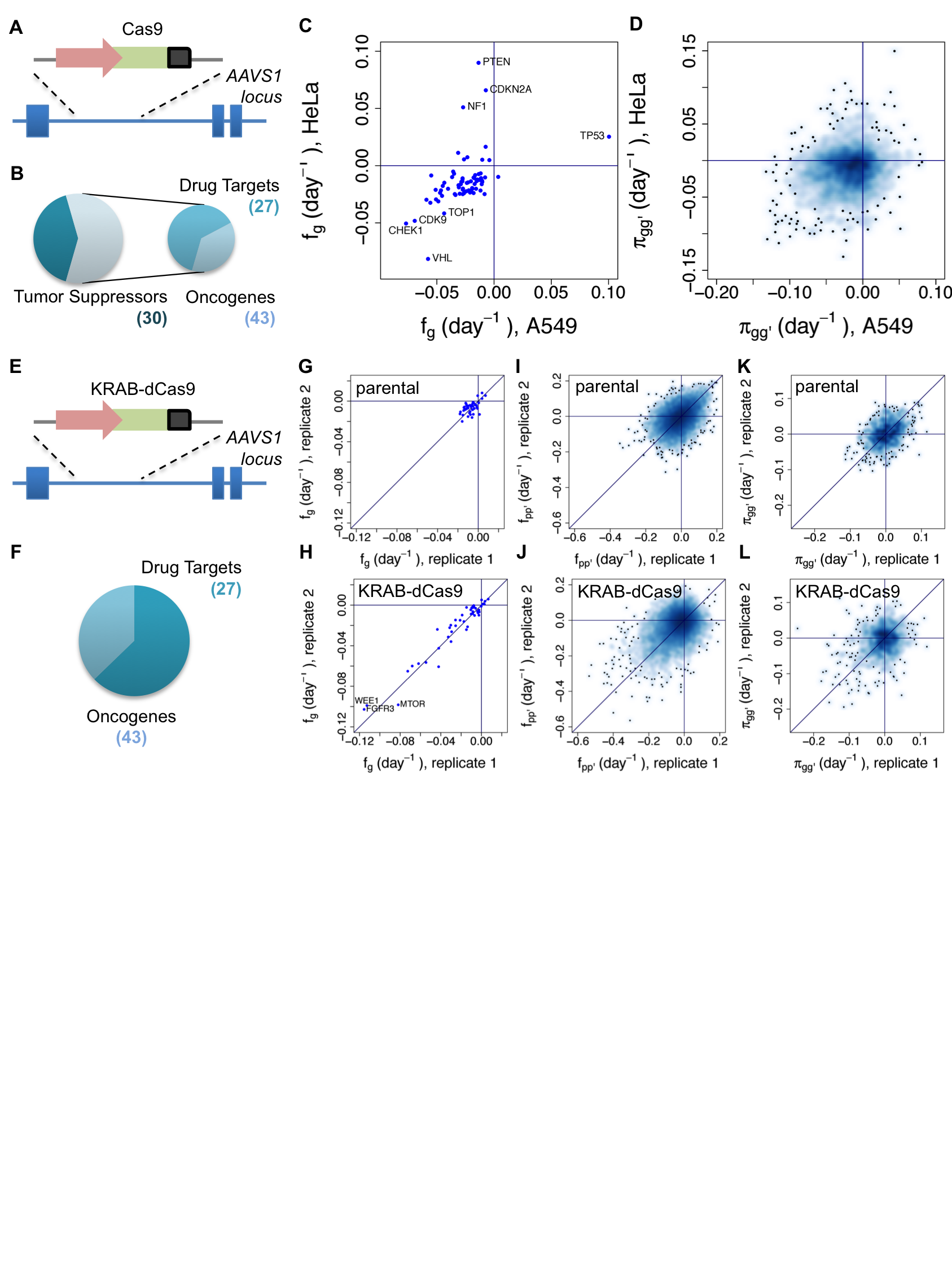
**Figure 1. Experimental and computational framework for elucidating genetic interactions with combinatorial CRISPR screens.**

**Figure 2. Mapping genetic interactions in cancer cell lines via CRISPR and CRISPRi screens.**

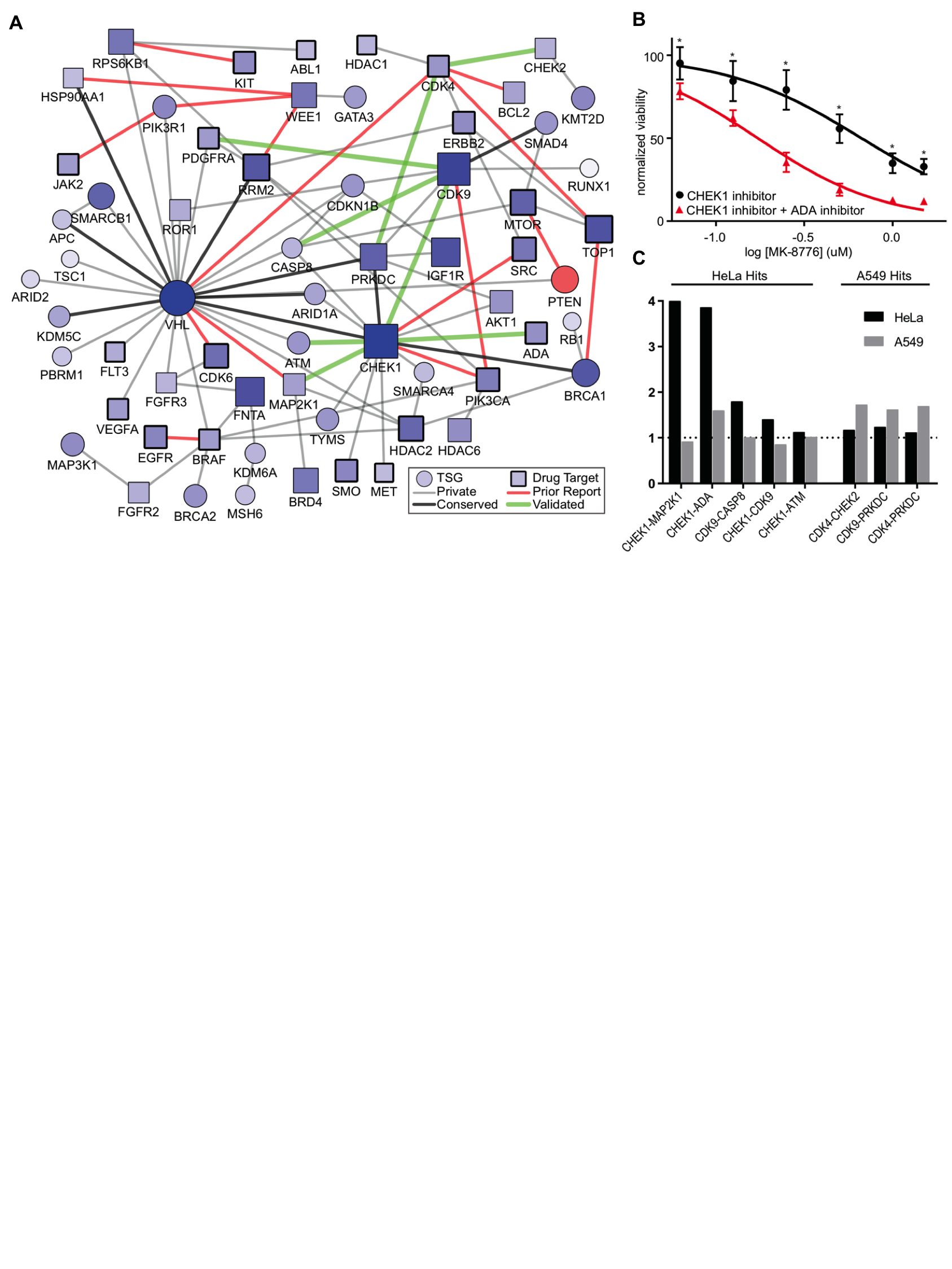
**Figure 3. Targeted validations of synthetic lethal interaction in cancer cell lines.**



**Figure 1.**

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**Figure 2.**

****

**Figure 3.**

# **METHODS**

**Dual gRNA library cloning**

The cloning of the dual gRNA library was carried out in two steps. The first step was to assemble the paired gRNAs into the backbone vector, and in next step a fragment including both the first gRNA scaffold and a mouse U6 promoter was inserted between the paired gRNAs.

*Step I: paired gRNA cloning*

The pooled oligonucleotide libraries were synthesized by CustomArray. Full-length oligonucleotides with dual gRNA spacers (i.e., 20-bp sequences used for targeting desired genes) were amplified by PCR using Kapa Hifi (Kapa Biosystems). The PCR reactions were set up according to manufacturer’s protocol, using 1 μl of synthesized oligonucleotide template (typically around 20 ng), an annealing temperature of 55°C, and an extension time of 15 s. The numbers of cycles were tested to fall within the linear phase of amplification. 28 cycles was used in this experiment. Sequences of the primers used were:

OLS\_gRNA-SP\_F: TATATATCTTGTGGAAAGGACGAAACACCG

OLS\_gRNA-SP\_R: CTTATTTTAACTTGCTATTTCTAGCTCT

To obtain a high yield and coverage of the PCR products, 10 repeats of 50 ul PCR reactions were performed for each library. The 144-bp amplicons were separated in 2% agarose gel electrophoresis and purified by QIAquick gel extraction kit (QIAGEN). Subsequently, the gRNA-LGP vector was digested by BsmBI (NEB) via the following reaction at 55°C for 3 hours:

|  |  |
| --- | --- |
| gRNA-LGP vector | 4 μg |
| Buffer 3.1 | 5 μl |
| 10× BSA | 5 μl |
| BsmBI | 3 μl |
| H2O | Up to 50 μl |

After digestion, the vector was treated with 2 μl of Calf Intestinal Alkaline Phosphatase (NEB) at 37°C for 30 minutes, then purified by QIAquick PCR Purification Kit (QIAGEN). To assemble the paired gRNAs into the vector, 10 Gibson assembly reactions were performed as follows:

|  |  |
| --- | --- |
| Linearized gRNA-LGP vector | 200 ng |
| Dual gRNA inserts | 36 ng (molar ratio 1:10) |
| 2× Gibson Assembly Master Mix (NEB) | 10 μl |
| H2O | Up to 20 μl |

After incubation at 50°C for 1 hour, the product was purified by QIAquick PCR Purification Kit (QIAGEN) and then transformed into One Shot Stbl3 Chemically Competent E. coli (Invitrogen). Twenty parallel transformations were performed to ensure several-fold library representation. A small fraction (20-100 μl) of cultures were spread on carbenicillin (50 μg/ml) LB plates to calculate the library coverage, and the rest of the cultures were amplified overnight in 150 ml LB medium. ~100X library coverage was ensured. The plasmid DNA was then extracted using HiSpeed Plasmid Maxi Kit (QIAGEN), and also twenty independent clones were picked and Sanger-sequenced to estimate the overall quality of the library.

*Step II: Insertion of the gRNA scaffold and the mouse U6 promoter*

The step-1 library plasmids were digested by BsmBI (NEB) as per the following reaction at 55°C for 3 hours:

|  |  |
| --- | --- |
| Step 1 library | 4 μg |
| Buffer 3.1 | 5 μl |
| 10× BSA | 5 μl |
| BsmBI | 3 μl |
| H2O | Up to 50 μl |

After digestion, the linearized plasmids were treated with 2 μl of Calf Intestinal Alkaline Phosphatase (NEB) at 37°C for 30 minutes; cut plasmids gel-purified via 0.6% agarose gel electrophoresis and QIAquick gel extraction kit (QIAGEN). Concurrently, the step 2 inserts (synthesized by IDT and cloned into a TOPO vector), were digested by BsmBI (NEB) per the following reaction at 55°C for 3 hours:

|  |  |
| --- | --- |
| Purified step 2 insert PCR product | 0.8 μg |
| Buffer 3.1 | 5 μl |
| 10× BSA | 5 μl |
| BsmBI | 3 μl |
| H2O | Up to 50 μl |

*Sequence of the step 2 insert, with left-gRNA scaffold (blue) and mU6 promoters (brown) highlighted:*

*TATGAGGACGAATCTCCCGCTTATACGTCTCTGTTTCAGAGCTATGCTGGAAACTGCATAGCAAGTTGAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTGTACTGAGTCGCCCAGTCTCAGATAGATCCGACGCCGCCATCTCTAGGCCCGCGCCGGCCCCCTCGCACAGACTTGTGGGAGAAGCTCGGCTACTCCCCTGCCCCGGTTAATTTGCATATAATATTTCCTAGTAACTATAGAGGCTTAATGTGCGATAAAAGACAGATAATCTGTTCTTTTTAATACTAGCTACATTTTACATGATAGGCTTGGATTTCTATAAGAGATACAAATACTAAATTATTATTTTAAAAAACAGCACAAAAGGAAACTCACCCTAACTGTAAAGTAATTGTGTGTTTTGAGACTATAAATATCCCTTGGAGAAAAGCCTTGTTTGAGAGACGGTACAAGCACACGTTTGTCAAGACC*

Subsequently the following ligation reaction was set up, and involved overnight incubation at 16°C followed by heat inactivation at 65°C for 10 minutes:

|  |  |
| --- | --- |
| 10X T4 DNA Ligase Buffer | 2 μl |
| Step 1 library, digested | 100 ng |
| Step 2 insert, digested | 100 ng |
| T4 DNA Ligase (high concentration) | 1 μl |
| H2O | Up to 20 μl |

4 ul of the reaction was transformed into 100 ul of ElectroMAX Stbl4 Competent Cells (Invitrogen) according to the manufacturer’s protocol using an Eppendorf Electroporator. A small fraction (1-10 μl) of cultures were spread on carbenicillin (50 μg/ml) LB plates to calculate the library coverage, and the rest of the cultures were plated on ten 15 cm LB- carbenicillin plates and grown overnight at 37°C for amplification. Two transformations were required to get ~100X library coverage. The plasmid DNA was extracted using HiSpeed Plasmid Maxi Kit (QIAGEN). Library diversity was determined by deep sequencing.

**NGS library preparation**

The harvested cell pellets were stored at -80°C until the genomic DNA was extracted with DNeasy Blood & Tissue Kit (QIAGEN). The dual gRNA cassette was amplified and prepared for deep sequencing using two steps of PCR. The 1st step PCR was performed as 10 separate 50 μl reactions with 2 μg input genomic DNA per reaction (total 20 μg for each sample) using Kapa Hifi. The 1st step PCR primers were as follows:

NGS\_dual-gRNA\_SP\_Lib\_F: ACACTCTTTCCCTACACGACGCTCTTCCGATCT TATATATCTTGTGGAAAGGACGAAACACCG

NGS\_dual-gRNA\_SP\_Lib\_R: GACTGGAGTTCAGACGTGTGCTCTTCCGATCT CCTTATTTTAACTTGCTATTTCTAGCTCTA

The thermocycling parameters were: 95°C for 30 s, 21-26 cycles of (98°C for 15 s, 55°C for 15 s, 72°C for 45 s), and 72°C for 5 min. The numbers of cycles were tested to fall within the linear phase of amplification. The amplicons (600 bp) of 10 reactions for each sample were pooled, size-selected and purified by Agencourt AMPure XP beads (ratio 0.8x), followed by a further purification using QIAquick PCR Purification Kit (QIAGEN). The 2nd step PCR was performed via 4 separate 50 μl reactions with 5 ng of 1st step PCR product per reaction (total 20 ng for each sample) using Next® Multiplex Oligos for Illumina (NEB) to attach Illumina adaptors and indexes. The thermocycling parameters were: 95°C for 30 s, 7-8 cycles of (98°C for 15 s, 72°C for 45 s), and 72°C for 5 min. The amplicons from these 4 reactions for each sample were pooled, size-selected and purified by Agencourt AMPure XP beads (ratio 0.8x) for twice. The purified 2nd step PCR library was quantified by real-time PCR using Illumina Library Quantification Kit (Kapa Biosystems) and used for downstream sequencing using the Illumina HiSeq Rapid run platform.

## **Cell culture**

HEK293T cells were maintained in DMEM medium supplemented with 10% fetal bovine serum. To produce lentivirus, HEK293T cells were seeded in 15 cm tissue culture dishes one day before transfection so that they were 70-80% confluent at the time of transfection. Prior to transfection, culture media was changed to pre-warmed DMEM medium supplemented with 10% fetal bovine serum. For each 15 cm dish, 36 ul of Lipofectamine 3000 (Life Technologies) was diluted in 1.2 ml OptiMEM (Life Technologies). Separately, 3 ug of pMD2.G (Addgene #12259), 12 ug of pCMV delta R8.2 (Addgene #12263), 9 ug of lentiviral vector and 48 ul of P3000 Reagent was diluted in 1.2 ml OptiMEM. After incubation for 5 min, the Lipofectamine 3000 mixture and DNA mixture were combined and incubated at room temperature for 30 min. The mixture was then added to HEK293T cells dropwise. Viral particles were harvested 48 hours and 72 hours after transfection, further concentrated using Centricon Plus-20 centrifugal ultrafilters with a cutoff 100,000NMWL (Millipore) to a final volume of 450 ul and then aliquot and frozen at -80°C.

Stable human cell lines HeLa and A549, in which the CRISPR Cas9 nuclease is stably integrated into the human AAVS1 site, were used for screening assays. The cells were expanded and frozen in multiple aliquots so that subsequent experiments could be performed on cells with low (less than 5) passage number. These were grown in DMEM supplemented with 10% fetal bovine serum and hygromycin (500 and 100 ug/ml, respectively). The puromycin selection doses were 5 ug/mL for both cell lines. Essentially 100% killing was observed in plain cells with these doses after 120 hours of exposure.

## **Design of gene constructs**

A panel of 73 genes comprising 17 validated oncogenes, 30 validated tumor suppressor genes and 26 cancer-relevant druggable targets were selected for study (**Table S1**). Priority was given to genes most frequently mutated in human cancer and to genes that are the target of an FDA-approved drug. For each gene, gRNAs were designed to target 20-bp sequences followed by NGG. A large number of gRNAs were designed to target the earliest exon of each gene and/or constitutive exons, as previously reported24. Poly-T sequences (i.e., more than two consecutive T's) were avoided, and to avoid off-target editing, gRNAs were only used if they were at least 3 base pairs away from any other location in the genome. After filtering all gRNA designs for the aforementioned criteria, three gRNAs were selected with one targeting the earliest exon and two targeting the earliest constitutive exons. Dual gRNA constructs were synthesized for all pairwise gRNA combinations between genes. In addition, twelve gRNAs were designed to be “non-targeters” that should not target any specific site in the genome. Three of these were randomly selected and paired with all targeting gRNAs to provide single knockout constructs. In addition, pairs of non-targeting gRNAs were included as negative controls. In total this resulted in 23652 double gene knockout constructs, 657 single gene knockouts, and 600 negative controls.

## **Experimental overview**

To demonstrate the utility of this approach, we conducted a negative selection screen by measuring the depletion of gRNAs targeting the pairs of oncogenes and tumor suppressor genes in two cancer cell lines, representing different tissue sources with different genetic bases for cancer. Specifically, we measured the impact of single and double knockouts in the HeLa and A549 cell lines, representing cervical carcinoma and lung adenocarcinoma respectively. All double gRNA constructs were packaged into lentiviruses and each cell line was infected at an MOI of 0.1-0.4 to ensure that each cell had 0-1 double gRNA constructs. In order to maintain adequate representation of all 23,652 elements of the library (minimum of 200-fold), the screen was started with 10 million cells for HeLa and A549 cells. In order to accommodate this larger number of cells, 500 cm2 bioassay plates (Corning) were used. Puromycin selection was started 2 days after transduction and maintained throughout the course of the experiment to eliminate cells without gRNAs. Following transduction, cells containing integrated gRNAs were maintained in exponential growth by harvesting and removing a fraction of the cells approximately every two to four days. A minimum of 5 million cells were maintained in culture for all cell lines at each passage. DNA was extracted from cells harvested at 3-, 14-, 21- and 28- day time points following transduction using Blood & Cell Culture DNA Mini Kit (QIAGEN) according to manufacturer recommended protocols. In order to assess the frequency of gRNAs before and after selection, the integrated DNA encoding the gRNA sequence was PCR-amplified and prepared for sequencing on a HiSeq Rapid run following manufacturer recommended protocols. Standard Illumina sequencing primers were employed for library prep, and sequencing was conducted, generating 75-bp reads in a paired-end fashion. Following sequencing, data quality was assessed using FastQC.

**Analysis**

Analysis was performed using a software pipeline constructed from Python, R, and Jupyter Notebook. In brief, FASTQ files provided by the sequencing center were first trimmed of scaffold sequence using the cutadapt ([ADD REFERENCE: Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.Journal, 17(1), pp. 10-12. doi:<http://dx.doi.org/10.14806/ej.17.1.200>]) program, after which those with either forward or reverse reads having trimmed lengths <19 or >21 bases were discarded. Remaining trimmed reads were truncated to 19 bases from the appropriate end, and reverse reads were reverse-complemented. Both reads in a pair were checked for sequence matches against gRNA sequences used in the library, allowing one mismatch anywhere in a read. Read pairs that matched to a known construct were aggregated to the total counts for that construct in the relevant sample, which were input to the analysis below as *mc.*

We assume that each sub-population of cells expressing a particular construct *c* grows independently of all others. In the continuous limit,

(1)

where is the number of cells in the population expressing construct *c* at time *t*, is the growth rate of cells expressing a double-null (control) construct, and is the *fitness* of construct *c*. As we measure time in days, fitness is measured in units of (day-1), as the number of doublings per day. We sample aliquots of cells from the population at time points and measure the abundances of sub-populations within each finite sample, which allows us to estimate *relative abundances* of cells in the population. It is convenient to define log2 relative abundances as  
These obey the following equations:

which have linear (in time) and non-linear components. Here is the initial condition. Note that while the same construct is expected to have the same fitness in replicated experiments, it may have different initial conditions. The non-linear term reflects the fact that relative frequency of one species is modulated by growth or decay of other species. Because of this, a particular may possibly decrease even when its fitness is positive. As we are working with relative frequencies, there is no need to “normalize” the sample counts in any way.

By definition, log2 relative frequencies satisfy the constraint at all times. Experimentally estimated log2 relative frequencies are derived from the construct counts:

In a model described by the set , the deviate from the exact values by

The parameters of the model are found by minimizing the sum of squares

subject to a constraint Since is invariant under the substitution where is an arbitrary constant, the fitnesses are determined up to an overall additive constant, which will be fixed by setting the mean null probe fitness to zero. Formally, one has to find the minimum of the function , where is the Lagrange multiplier. In other words, we need to solve the system of non-linear equations

An analytical solution does not exist; however, an excellent approximation exists when the number of constructs is large, , which is the case for large screens. In this case

and

and the solution is

and

where the bar indicates mean over time points. do not depend on the choice of .

Once are known, we find the probe-level fitnesses and probe-level interactions as follows. As each construct contains two gRNA probes, and , we write

(2)

where is the probe-level interaction. As there are “genes” in the CV4 panel (73 genes and one null “gene”), each represented by three distinct probes, there are a total of constructs. Each probe is effectively replicated times as it appears in as many equations. The probe-level -scores are as unique as the construct fitnesses The probe fitnesses are found by robust fitting of Eq. (2). The probe-level -scores are the residuals of the robust fit.

We have found that inclusion of all data points in fitting leads to an unacceptably large variance in between replicates. The problem can be traced to data points from undersampled constructs, and disappears when we use only points above a certain threshold (**Supplementary Figure 4**). The threshold depends mainly on the size of the sample (number of cells) collected at a given time in relation to the size of the viral library and on the depth of sequencing. We note that the left-most peak in the histograms of **Supplementary Figure 4** contains constructs with zero counts in the sequencing. Their coordinate corresponds to a pseudo-count of 1 introduced only to visualize this sub-population in the histogram plots. It is arbitrary and therefore should not be used for fitting the model. By extension, very low count values should also be viewed as non-representative and should be dropped from the model. We set a threshold for every time point (red lines in **Supplementary Figure 4**), below which the data are ignored during fitting. The thresholds chosen are admittedly somewhat subjective but they are chosen to be as low as possible while keeping the variance artifacts under control. Note also that the right tail of these histograms moves to the right with time – this is due to the fastest-growing subpopulations taking progressively larger fraction of the cells sampled. Also related is the fact that the peak of zero counts becomes taller as smaller sub-populations are outcompeted by faster growing ones and thus tend to become undersampled.

**Supplementary Figure 5A** shows a replicate plot of probe-level fitnesses. Each gene is represented by three probes. We highlight three “genes” – the null gene as well as two genes with large positive and negative probe fitnesses. The origin is set to the center of mass of the null probes by a choice of . Reassuringly, the null probes cluster closely together. It is true of almost all genes that one of the three probes is not performing very well and that the median probe splits the difference. In order to not dilute the signal with underperforming probes, we rank the probes as in ascending order of . The ranks define weights for averaging as follows: the gene-level fitnesses are calculated as the weighted means

and

The sums are over probes that represent genes g and g’. The weights are designed so that probes with rank 0 do not contribute to the means, and the “best” probes have highest weights. This way, each gene-level fitness is determined by two probe-level fitnesses, and each gene-level interaction is determined by four probe-level interactions. Note that this ansatz may not be appropriate for other probe designs: for instance, if all three probes performed well, it might be appropriate to choose equal weights. Finally, we must keep in mind that each probe-level fitness has effectively 219 replicates, but each probe-level interaction has only one. It is therefore imperative that the set of construct fitnesses be determined with maximum possible accuracy.

Example fits are shown in **Supplementary Figure 6**. In the top panels, the fitted agrees well between replicates, but only after undersampled points had been dropped. In the bottom panels, we show examples when does not agree well between replicates despite no obvious undersampling. These cases come in two flavors: those in which the measured data have large variance (bottom left) and those in which the data have clear trends in both replicates but they disagree (bottom right). In the latter case, we do not know whether there is a real biological difference between replicated experiments or whether this is just a fortuitous ordering of four data points with large variances into apparent trends. The way to properly resolve these questions would be with more replicates and more time points. Perhaps this will be feasible in the future, but at the present time we decided to take this variance at face value and incorporate it into a model that presumably increases power by using data from both replicates.

In this model, we do not look for separately for each replicate. Rather, we find a single optimal from data points ( minus any number of points below the threshold). As noted above, here we assume that does not change between experiments but that the initial conditions may be different in each replicate. Each comes with a raw p-value calculated from the -statistic

where

is the standard error of . Factor is the number of degrees of freedom. With time points and two replicates, is an integer between 3 and . The raw p-values are transformed into posterior probabilities using theory of Storey25,26, which connects frequentist -values with Bayesian posterior probabilities in the context of the two-groups model. We find that roughly 2/3 of the posterior probabilities are zero, which means that about 2/3 of ’s in Eq. (2) are spurious and are likely truly zero. We avoid fitting to noise by designing a numerical Bayesian ensemble of experiments: In each member of the ensemble we assign a fitness value to construct , which is either 0 with probability , or a random Gaussian-distributed number with mean and standard deviation . The latter value of standard deviation includes both the experimental variance from sampling and counting as well as possible biological variance. All cell subpopulations are made explicitly statistically independent, which is true if they are not interacting as expressed by Eq. (1). We typically create samples, calculate gene-level quantities and for each ensemble member, and report ensemble mean values and other statistics. We think the above sampling procedure is a reasonable data-driven solution to the bias-versus-variance problem. Finally, we consider interaction to be a candidate for further validation if it has a large absolute *z*-score, typically

**References**

1. Krogan, N.J., Lippman, S., Agard, D.A., Ashworth, A. & Ideker, T. The cancer cell map initiative: defining the hallmark networks of cancer. *Mol Cell* **58**, 690-8 (2015).

2. Hofree, M. *et al.* Challenges in identifying cancer genes by analysis of exome sequencing data. *Nat Commun* **7**(2016).

3. Luo, B. *et al.* Highly parallel identification of essential genes in cancer cells. *Proc Natl Acad Sci U S A* **105**, 20380-5 (2008).

4. Skalamera, D. *et al.* A high-throughput platform for lentiviral overexpression screening of the human ORFeome. *PLoS One* **6**, e20057 (2011).

5. Wang, T. *et al.* Identification and characterization of essential genes in the human genome. *Science* (2015).

6. Vizeacoumar, F.J. *et al.* A negative genetic interaction map in isogenic cancer cell lines reveals cancer cell vulnerabilities. *Mol Syst Biol* **9**, 696 (2013).

7. Whitehurst, A.W. *et al.* Synthetic lethal screen identification of chemosensitizer loci in cancer cells. *Nature* **446**, 815-9 (2007).

8. Bassik, M.C. *et al.* A systematic mammalian genetic interaction map reveals pathways underlying ricin susceptibility. *Cell* **152**, 909-22 (2013).

9. Horn, T. *et al.* Mapping of signaling networks through synthetic genetic interaction analysis by RNAi. *Nat Methods* **8**, 341-6 (2011).

10. Laufer, C., Fischer, B., Billmann, M., Huber, W. & Boutros, M. Mapping genetic interactions in human cancer cells with RNAi and multiparametric phenotyping. *Nat Methods* **10**, 427-31 (2013).

11. Mali, P. *et al.* RNA-guided human genome engineering via Cas9. *Science* **339**, 823-6 (2013).

12. Cong, L. *et al.* Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819-23 (2013).

13. Jinek, M. *et al.* RNA-programmed genome editing in human cells. *Elife* **2**, e00471 (2013).

14. Mali, P., Esvelt, K.M. & Church, G.M. Cas9 as a versatile tool for engineering biology. *Nat Methods* **10**, 957-63 (2013).

15. Kabadi, A.M., Ousterout, D.G., Hilton, I.B. & Gersbach, C.A. Multiplex CRISPR/Cas9-based genome engineering from a single lentiviral vector. *Nucleic Acids Res* **42**, e147 (2014).

16. Gilbert, L.A. *et al.* Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. *Cell* **159**, 647-61 (2014).

17. Shalem, O. *et al.* Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science* **343**, 84-7 (2014).

18. Wang, T., Wei, J.J., Sabatini, D.M. & Lander, E.S. Genetic screens in human cells using the CRISPR-Cas9 system. *Science* **343**, 80-4 (2014).

19. Konermann, S. *et al.* Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature* **517**, 583-8 (2015).

20. Baryshnikova, A., Costanzo, M., Myers, C.L., Andrews, B. & Boone, C. Genetic interaction networks: toward an understanding of heritability. *Annu Rev Genomics Hum Genet* **14**, 111-33 (2013).

21. Manic, G., Obrist, F., Sistigu, A. & Vitale, I. Trial Watch: Targeting ATM–CHK2 and ATR–CHK1 pathways for anticancer therapy. *Molecular & Cellular Oncology* **2**, e1012976 (2015).

22. Choi, M., Kipps, T. & Kurzrock, R. ATM Mutations in Cancer: Therapeutic Implications. *Mol Cancer Ther* **15**, 1781-91 (2016).

23. Wong, A.S.L. *et al.* Multiplexed barcoded CRISPR-Cas9 screening enabled by CombiGEM. *Proceedings of the National Academy of Sciences of the United States of America* **113**, 2544-2549 (2016).

24. Sanjana, N.E., Shalem, O. & Zhang, F. Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods* **11**, 783-4 (2014).

25. Storey, J.D., The positive false discovery rate: a Bayesian interpretation and the q-value, *The Annals of Statistics*, 31, **6**, 2013–2035 (2003).

26. Storey, J.D. with contributions from Bass, A.J. Bass, Dabney A. and Robinson D, qvalue: Q-value estimation for false discovery rate control. R package version 2.4.2. [*http://github.com/jdstorey/qvalue*](http://github.com/jdstorey/qvalue)(2015).