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| **Supplementary Figure 1** |
| Schematic of cloning strategy for dual-gRNA library synthesis. |
| 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. |
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| **Supplementary Figure 2** |
| Evaluation of activity of engineered gRNA scaffolds. |
| **(a)** A range of mutagenized gRNA scaffold sequences (designed to increase sequence diversity but maintain the primary hairpin loops in the gRNA scaffold via utilization of G-C vs. A-U interactions) were evaluated. **(b)** To assay their relative activity, NHEJ mediated mutagenesis of the AAVS1 locus was performed in HEK 293T cells and editing rates quantified 1-week post transduction of gRNA constructs. Two adjacent targets T1 and T2 (sequences labeled respectively in red and green) were targeted via corresponding gRNAs. Representative next generation sequencing results are shown, bar plots indicate ratio of reads showing indel relative to total reads. These experiments indicated that the wild-type scaffold (version 1) and an engineered scaffold (version 4) were the most active and showed the most consistent activity. **(c)** The effect of position of a gRNA in a dual-gRNA construct was evaluatedvia its ability to induce NHEJ mediated mutagenesis of EGFP loci in a HEK 293T cell line stably expressing the protein. In these experiments loss of GFP expression was assayed 96 hours post transduction of dual-gRNA constructs. The percentage GFP+ cells are indicated in the FACS panel showing side scatter (SSC) vs. GFP signal. Bar graph shows average with error bar representing +/- SD from three technical replicates. These experiments confirmed that the two gRNAs positions in the targeting construct were equally functional (left: hU6-gRNA; right: mU6-gRNA). **(d)** Similarly the simultaneous activity of two gRNA in the same construct was evaluated by measuring loss of EGFR and mCherry expression in a HEK 293T cell line stably expressing both proteins. The percentage GFP+ and mCherry+ cells are indicated in each bar graph, error bar represents +/- SD from three technical replicates. Below is representative FACS panel showing mCherry vs. EGFP. |
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| **Supplementary Figure 3** |
| Multiple time point Regression with Bayesian sampling analysis method. |
| **(a)** Samplehistograms of log2 relative frequencies of dual-gRNA constructs for HeLa cells**.** Red lines denote abundance cutoffs below which the data is deemed under-sampled and dropped. Note that after 28 days of selection there is a broader distribution with more gRNA falling under the threshold. **(b)** Representative data for fitting of a few selected gRNA constructs for HeLa cells. Black and blue points and lines correspond to data points and continuous fits for replicates 1 and 2, respectively. Empty symbols denote data points below the abundance threshold, which are not used for fitting. The slight curvature to the fitted lines is due to the nonlinear term in Eq. 3. **(c)** Comparison of unique gRNA probes for each gene for HeLa cells. The three gRNA probes targeting each gene are not equally active, however the fitness effect of each gRNA was highly correlated between replicates. Here the three gRNA for PTEN, VHL and the non-targeting are highlighted, showing that only two of the three gRNA have an effect greater than that of the non-targeting gRNA probes. These observations motivated our efforts to rank individual probes by activity and accordingly weight their effects in the overall analyses. **(d)** Kernel density plot for CRISPR-Cas9 screen probe ranks across screened genes for both HeLa and A549 cell lines. Red line indicates orthogonal regression result. Probe ranks assigned to each gene in both screens correlate significantly (Pearson r = 0.42, ). |
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| **Supplementary Figure 4** |
| Replicate analyses and cell line comparisons. |
| Scatterplot of fitness (*fg*) for single gene knockouts forbiological replicates in **(a)** HeLa, **(b)** A549, and **(c)** 293Tcells. Scatterplot of raw genetic interaction scores () for biological replicates in **(d)** HeLa, **(e)** A549, and **(f)** 293Tcells. The significant interactions underlying the correlation calculation are highlighted in bold**.** **(g)** Genes that have large fitness effects when they are disrupted have greater number of genetic interactions (node degree, includes positive interactions as well as synthetic lethal interactions). This was true for Hela (blue), A549 (pink) and 293T (black) cells. Interaction hubs (genes with highest node degree) are highlighted for each cell line. **(h)** Scatterplot of fitness (*fg*) for single gene knockouts in HeLa versus 293T. **(i)** Scatterplot of fitness (*fg*) for single gene knockouts in A549 versus 293T. **(j)** Scatterplot of interaction scores in HeLa versus 293T. **(k)** Scatterplot of interaction scores in A549 versus 293T. |
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| **Supplementary Figure 5** |
| Single gene fitness effects relative to gene expression. |
| Scatterplot of expression vs. single gene fitness effect (fg) for **(a)** HeLa, **(b)** A549, and **(c)** 293T cells. Pearson correlation r values and *p* values are indicated. |
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| **Supplementary Figure 6** |
| Single cell line synthetic lethal networks. |
| Z-score vs. False Discovery Rate (FDR) plots for **(a)** HeLa, **(b)** A549, and **(c)** 293Tcells. Synthetic lethal networks for **(d)** HeLa, **(e)** A549, and **(f)** 293T cells.Circles indicate TSG, squares druggable targets, squares outlined in bold indicate FDA approval of drug targeting that gene. Color of node indicates single gene knockout fitness effect, red: positive fitness effect, blue: negative fitness effect. Previously reported interactions are indicated as red edges, and interactions validated in drug-drug assays in this study are shown in green. Black edges were identified in multiple cell lines in this study. |
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| **Supplementary Figure 7** |
| Selection of ‘drug 2’ concentrations. |
| Single drug dose-response curves are shown for all drugs used as ‘drug2’ in a drug1-drug2 assay described in the manuscript: **(a)** trametinib **(b)** 5-FU **(c)** fludarabine **(d)** KU-60019 **(e)** dinaciclib **(f)** hydroxyurea **(g)** NU7441. X-axis is log10 of the concentration of the indicated drug in micromolar units. In drug1-drug2 assays, concentration of drug2 was set at the IC20 value (dotted line). Viability normalized to solvent-only controls independently for HeLa and A549 cells. Error bars represent +/- SD. |
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| **Supplementary Figure 8** |
| Validation of synthetic lethal interactions in drug-drug assays. |
| Dose-response curves for inhibitors of the following gene pairs **(a)** CHEK1-MAP2K1 **(b)** CHEK1-TYMS **(c)** ADA-CHEK1 **(d)** ATM-CHEK1 **(e)** CDK9-CHEK1 **(f)** PRKDC-RRM2 **(g)** CDK9-PRKDC **(h)** CDK4-PRKDC; HeLa on left and A549 on right of each pair; error bars represent +/- SD. X-axis is log10 of the concentration of the indicated drug in micromolar units. Gray curves show single agent dose-response of Drug#1 and are normalized to solvent control. Pink curves show dose response in presence of fixed concentration of Drug#2 and are normalized to effect of Drug#2 at indicated dose. To assess for synthetic lethal interaction, the IC50 values for gray and pink curves are compared for each plot using the sum-of-squares F test; visually the IC50 concentration is the point along the x-axis at which the curve crosses 50% viability (dashed line). When the pink curve crosses the 50% viability to the left of the gray this indicates a synergistic, or synthetic lethal interaction; *p* values are indicated with significant interactions (*p* < 0.05) highlighted in red. **(i)** Summary of synthetic-lethal interactions re-tested in arrayed drug-drug viability assays. **(j)** Matrix summarizing results from drug-drug validation assays for HeLa and A549 cell lines. A total of 8 gene pairs were each tested in both HeLa and A549 cells for a total of 16 tests. |