Inventory of Supplemental Information

Figure S1. Related to Figure 1

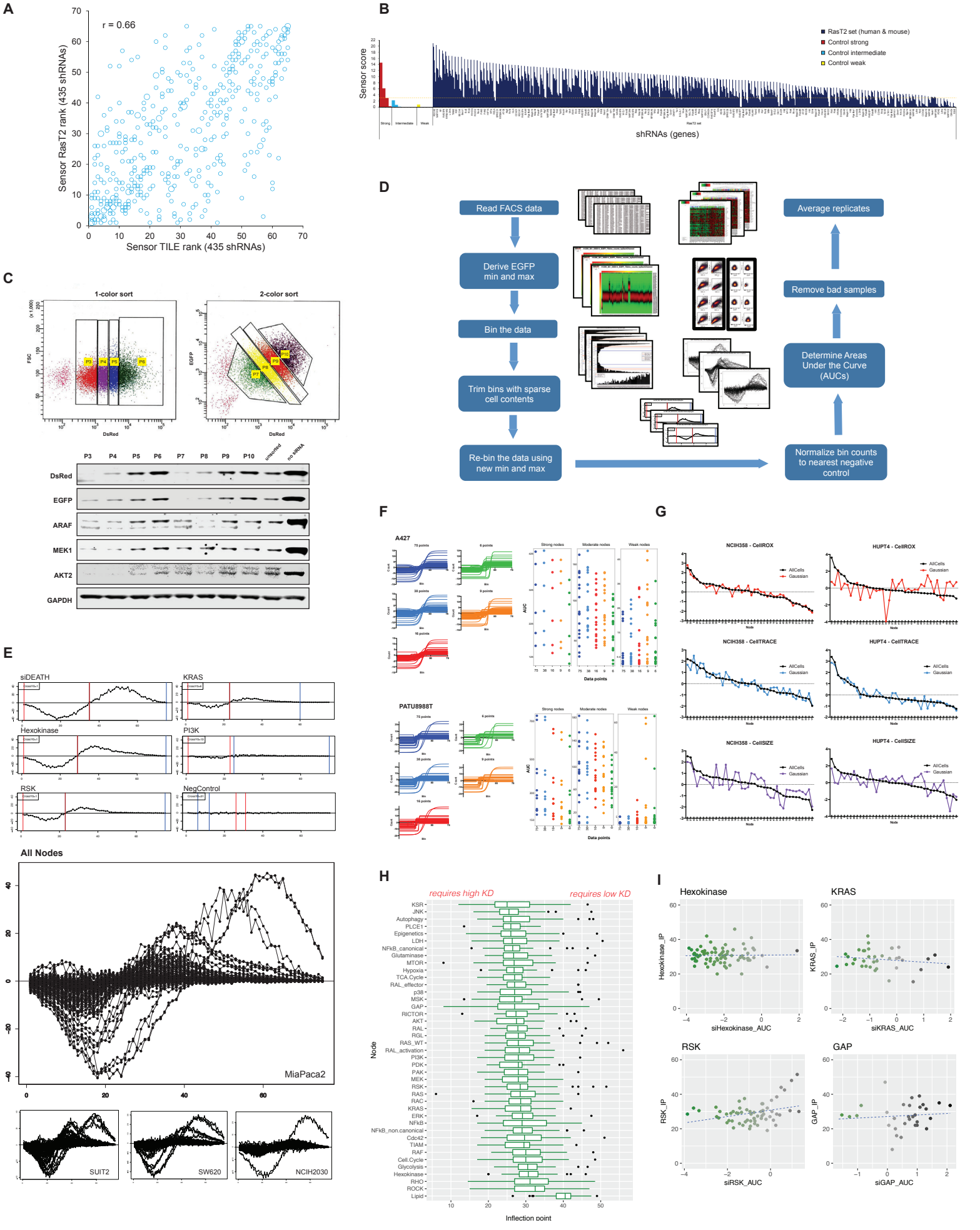


Figure S1. Related to Figure 1. Features of the siREN assay. (A) Sensor robustness and accuracy. Shown is the rank correlation of 435 performance control shRNAs that had previously been tested using the Sensor assay as part of the original TILE data set. The individual shRNA ranks between the TILE and RasT2 sets were highly correlated (Spearman rank correlation coefficient rho: 0.66). (B) Sensor score distribution of the top five Sensor identified shRNAs for each of the 166 genes. The dotted line indicates a threshold for potent shRNAs, based on known controls. Control shRNAs are five-fold overrepresented for better visualization. (C) DsRed and EGFP-expressing cells were transfected with a combination of Sensor siRNAs targeting DsRed, EGFP, ARAF, MEK1 and AKT2. Cells were sorted into 4 populations based on knockdown of one marker (DsRed) or two markers (DsRed and EGFP). Sorted cells were analyzed by western blot to detect the level of knockdown of all targets. Tracking the knockdown of just one marker was sufficient to report the level of knockdown of all intended targets. (D) Schematic of the derivation of the viability metric, AUC. See Supplemental Methods for details. (E) Raw drop-out/enrichment data from MiaPaca2 cells treated with siRNA against select nodes are shown, top panels. AUC curves for all tested nodes are overlaid, middle panel. Overlaid AUC curves for 3 cells lines: SUIT2 (sensitive to many nodes), SW620 (sensitive to few nodes) and NCI-H2030 (insensitive to most nodes) illustrate the range of node-sensitivity in the cell line panel. (F) ~30,000 cells with different levels of EGFP knockdown were analyzed per sample and were binned into 6, 9, 16, 38 or 75 EGFP-expressing bins. AUCs were determined for curves from each binning simulation. As with traditional dose-response curves, more bins (i.e. “doses”) gave greater resolution in AUCs. With 75 bins, a greater dynamic range was achieved among strong nodes, and false positives were minimized among weak nodes. (G) Phenotypic readouts were measured as the average of the entire population (black points), including untransfected, transfected and dead cells, or only in the population representing transfected cells (colored points). Measurements taken from all cells masked the true outcome of knockdown in the Gaussian population. (H) Analysis of viability across 75 EGFP bins (equivalent to 75 doses of node inhibition), allowed for the careful detection of the “inflection point”, or the level of knockdown at which viability begins to be compromised. Nodes that require less knockdown to achieve loss of viability may be more feasible drug candidates. (I) There is no correlation between dependency (AUC) and inflection point (IP), suggesting that node dependency is not contingent on the amount of target inhibited. Rather a threshold of target inhibition must be surpassed in order to see any degree of node dependency across dependent cell lines. Interestingly, the degree of target knockdown of tumor suppressive nodes, such as GAP, show more variability in IP, perhaps reflecting higher complexity in the signaling of growth-promoting activities.

Figure S2. Related to Figure 2 and Figure 3

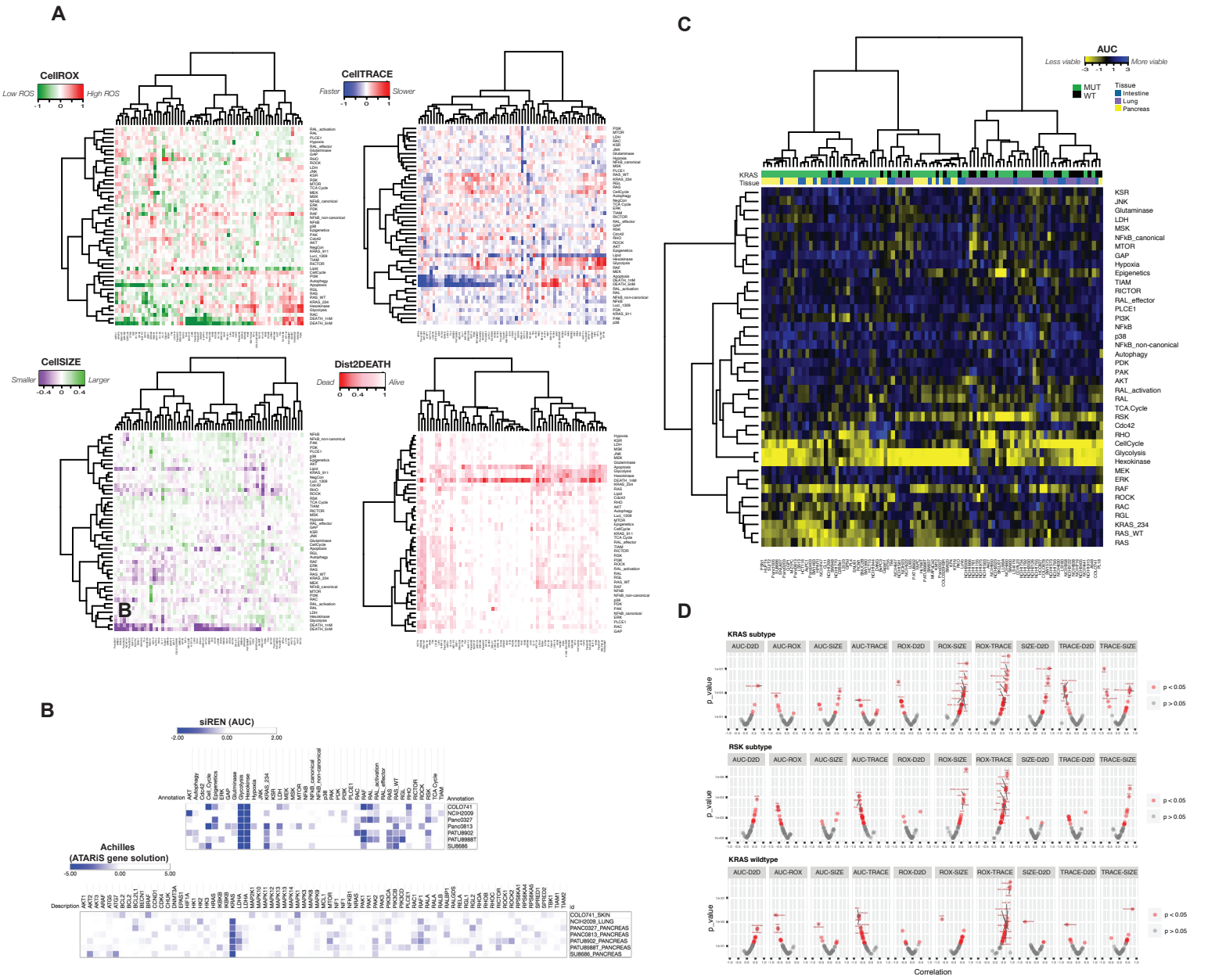


Figure S2. Related to Figure 2 and Figure 3. Details of siREN results. (A) Phenotypic effects of node knockdown in KRAS mutant lines are shown with hierarchical clustering of all cell lines and all nodes. (B) We compared the results of node knockdown (siREN) and single gene ablation (CRISPR) in 7 cell lines screened in common between our screen and a recently published CRISPR screen (Aguirre et al, 2016). Among all the genes in the siREN library, only KRAS causes a major viability effect when knocked out as a single gene by CRISPR, whereas KRAS is one of many nodes that lead to a big effect on viability when targeted as a complete node. (C) The effect of node knockdown on viability (AUC) across all nodes and all lines is shown. AUC values were not obtained from the Apoptosis node, given that no $EGFP_{low}$ cells remained after 96h. (D) Pairs of phenotypic parameters often correlate with each other, indicating a relationship between 2 biological responses to node knockdown. Nodes with significant correlations (Pearson correlation, t-test) between pairs of phenotypic parameters are shown. (E) KRAS- and RSK-type lines were treated with 6801640, SCH772984, AZD8055, OSU03012, Rotenone or DMSO for 72h and analyzed by western blot.

Figure S3. Related to Figure 4

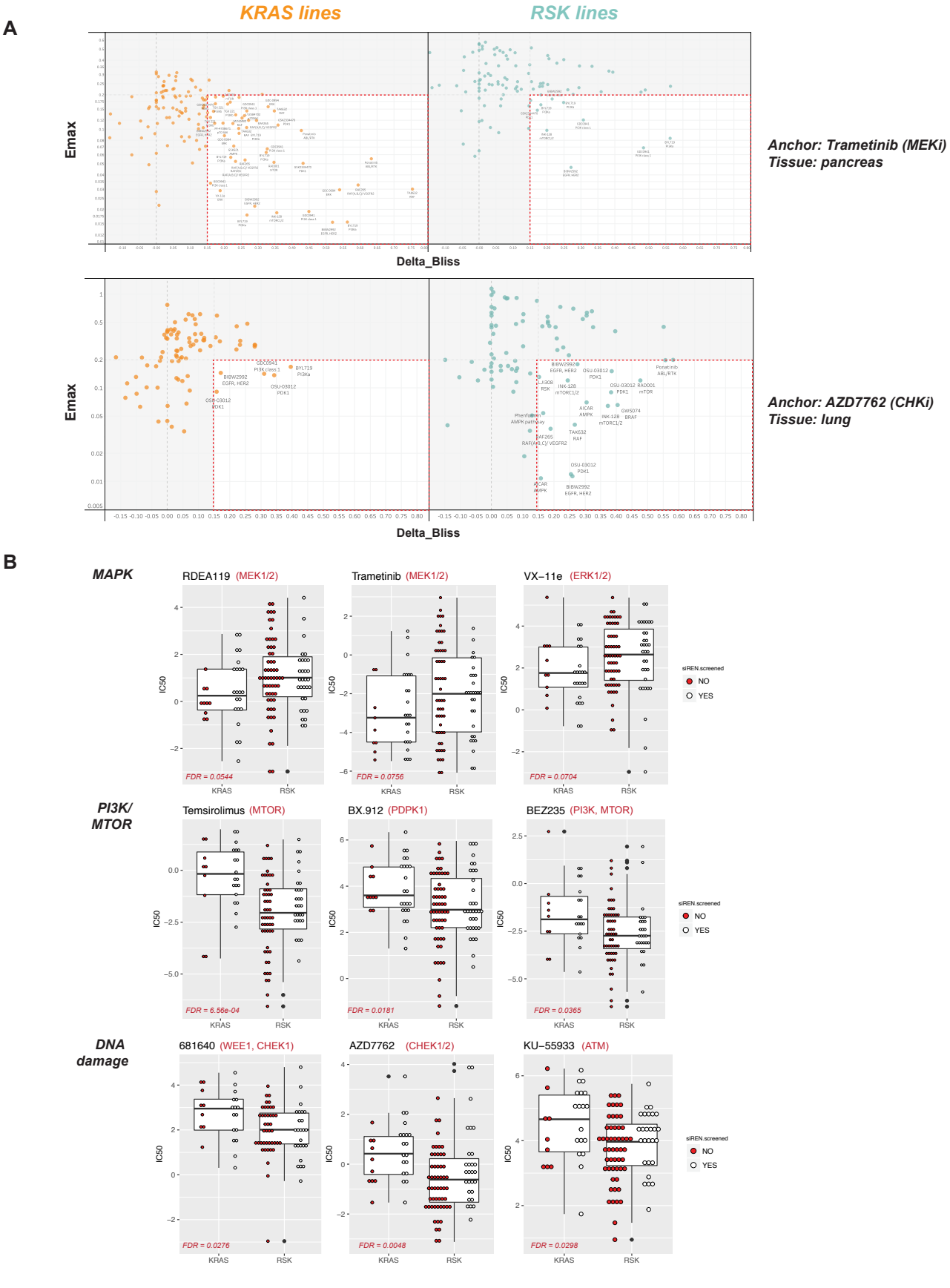


Figure S3. Related to Figure 4. (A) Synergistic combinations (low Emax, high delta_Bliss, highlighted in the red dashed box) with trametinib in pancreatic cell lines or AZD7762 in lung cell lines are shown. More synergistic combinations were identified in KRAS-type lines with trametinib as an anchor drug, while more synergistic combinations were identified in RSK-type lines with AZD7762 as an anchor drug. (B) KRAS-RSK_sig was used to stratify all cell lines screened in a study by Iorio F, et al (2016) into KRAS- and RSK-type bins. The IC50 values for each bin were plotted for drugs targeting MAPK, PI3K/MTOR and DNA damage pathways. As predicted by the single agent drug screen in Figure 3, KRAS-type lines were more sensitive to MAPK-targeting compounds, while RSK-type lines were more sensitive to PI3K/MTOR and DNA damage inhibitors.

Figure S4. Related to Figure 5

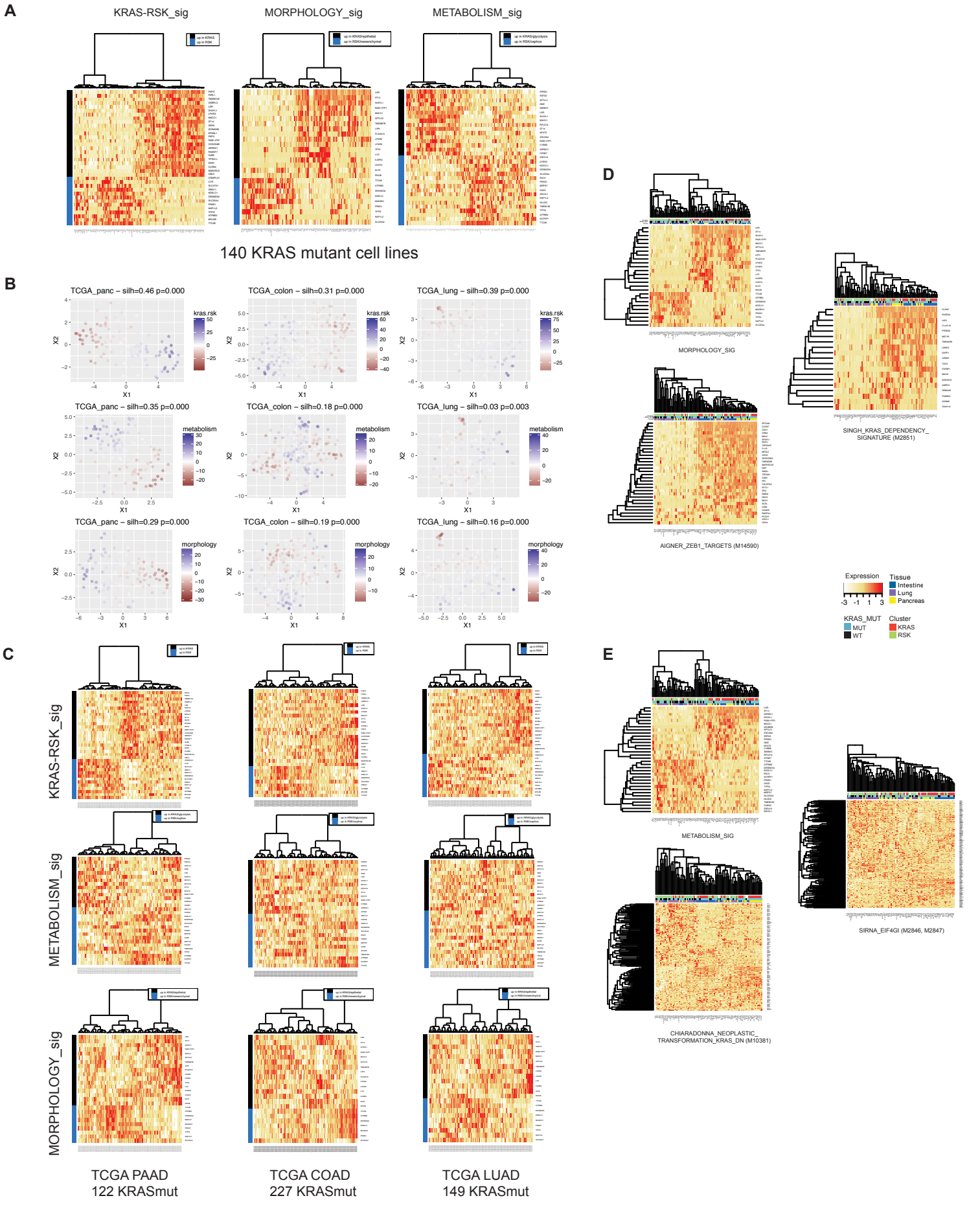


Figure S4. Related to Figure 5. Stratification of cell line and TCGA samples using EN-derived signatures. (A) The three signatures were projected onto 140 KRAS mutant cell lines. (B-C) KRAS mutant TCGA samples from lung, pancreatic and colorectal adenocarcinomas were analyzed using KRAS-RSK_sig, Morphology_sig and Metabolism_sig. Each dataset was split into two clusters according the each gene signature and tSNE transformed, as shown in (B). Euclidean distances were used to compute an average silhouette score and randomized 100,000 times to obtain a p-value. (D-E) The 92 cell lines screened in the siREN assay were stratified based on 3 EMT signatures and 3 metabolic signatures. All six signatures were able stratify most KRAS-type lines from RSK-type lines.

Figure S5. Related to Figure 6

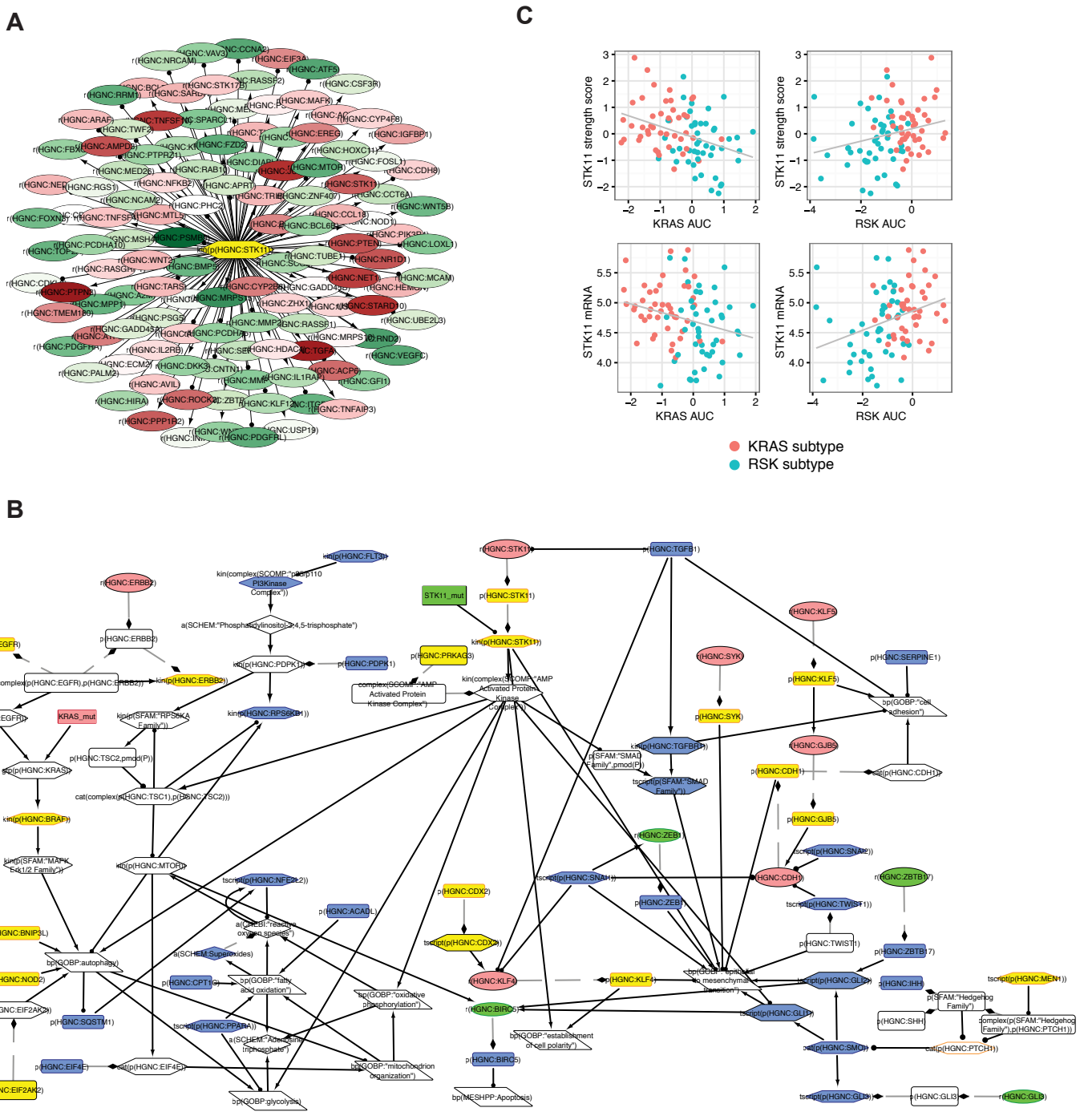


Figure S5. Related to Figure 6. (A) STK11 kinase activity is inferred from literature-based gene expression changes that result from STK11 perturbation. Genes are colored in shades of red and green to indicate a continuum of correlation (Pearson) values with KRAS sensitivity. Edges indicate increasing (arrows) and decreasing (solid circles) regulatory relationships between STK11 activity and downstream genes, as reported in the literature. All inferred mechanisms are evaluated within this type of infrastructure. Notations employ the Biological Expression Language (BEL), where "r" indicates mRNA of the indicated gene (in the HGNC namespace), and "kin" indicates the kinase activity of the indicated protein, "p". (B) STK11 mRNA expression and inferred kinase activity (STK11 strength score) were plotted against KRAS and RSK AUCs. High STK11 expression and activity correlate with KRAS-dependence while low STK11 expression and activity correlate with RSK-dependence. (C) Detailed Inferred Mechanistic Model of KRAS and RSK Phenotypes. Mechanisms were found to be significant if they were correlated or anti-correlated with sensitivity to KRAS or RSK AUC with a Pearson correlation FDR-corrected p-value <0.1, or if there was a strictly positive frequency by Elastic Net (EN) analysis. EN results largely overlapped with univariate Pearson correlation analysis results. Univariate correlation significance of inferred mechanisms is indicated by node fill: Blue - Inferred mechanism strength scores significantly correlated with RSK sensitivity or anti-correlated with KRAS sensitivity; Yellow - Inferred mechanism strength scores significantly correlated with KRAS sensitivity or anti-correlated with RSK sensitivity. EN-specified nodes are indicated by node outline: Orange – KRAS-sensitive or RSK-resistant mechanisms; Dark blue – RSK-sensitive or KRAS-resistant mechanisms. White nodes either did not reach significance or cannot be scored based on available knowledge, but serve to represent the likely paths given the inferred mechanism signal. RNA levels and genomic features were also directly assessed in the same fashion as mechanisms, and key observations from these analyses are also indicated within the model: Green fill - Gene expression measurements or genomic features (mutations) that significantly correlated with RSK sensitivity or anti-correlated with KRAS sensitivity; Red fill - Gene expression measurements or genomic features that significantly correlated with KRAS sensitivity or anti-correlated with RSK sensitivity; Dark green outline - RSK-sensitive or KRAS-resistant gene expressions or genomic features by EN; Dark red outline – KRAS-sensitive or RSK-resistant gene expressions or genomic features by EN. All edges between mechanisms are supported by literature evidence. Bold edges indicate specifically a direct action (e.g., involving protein-protein interaction or a protein binding a gene promoter). Dashed edges specify other types of relationships such as "translated to" (an edge from an mRNA to its protein) or "component of" a complex. Labels are in the Biological Expression Language (BEL) format, which indicates a function or activity and the name (in a specific namespace) of what is active: function(namespace: entity). Functions are indicated as follows: a=abundance, p=protein abundance, bp=biological process, r=RNA abundance, kin=kinase activity, gtp=GTP-bound activity, tscript=transcriptional activity, cat=catalytic activity, pmod=protein modification. Namespaces are indicated as follows: HGNC=HUGO Gene Nomenclature Committee, GOBP=GO biological process, SCHEM=Selventa chemical namespace, SFAM=Selventa Protein Families, SCOMP=Selventa named complexes. The individual inferred mechanisms of this model collectively inform an overall process model of RSK- and KRAS-sensitive phenotypes (Figure 5B) that is comprised of both processes that have been experimentally supported in this study and those that are proposed as key elements of the phenotypes.

Supplemental Title and Legends

Table S1. Related to Figure 1. Sensor assay results. The RasT2 Sensor set includes 83 human and 83 mouse genes (65 shRNAs/gene). Additionally, 27 controls (Cc) were spotted 2X on the chip for a total of 10817 oligos. V gmean, S3 gmean, S5 gmean and geometric mean of normalized sequencing read numbers of replicates in the vector library (V) after Sensor sort 3 (S3) and after Sensor sort 5 (S5) are reported. For details, see Fellmann et al., 2011 and Yuan et al., 2014.

Table S2. Related to Figures 1&3. siREN assay reagents. KRAS siREN library: siRNA sequences and percent knockdown (as measured by qPCR in U2OS cells) are provided. Sequences with inadequate knockdown by Sensor siRNA were substituted with sequences from Qiagen. Cell lines: cell line names and media conditions of all lines tested are provided. Miscellaneous reagents: qPCR primers and antibodies are provided.

Table S3. Related to Figures 2-6. Complete siREN dataset. Raw values were normalized by z-score across all nodes within each cell line.

Table S4. Related to Figures 3&5. **Gene expression analysis.** Differential gene expression between KRAS- and RSK-type lines is reported. Elastic Net (EN) results are summarized and EN-derived gene signatures are provided. EN features are *predictors* and the AUC of the indicated Node is the *response*. A positive Effect indicates that a high predictor value is associated with a high response value (high AUC = resistance to Node knockdown). A negative Effect indicates that a high predictor value is associated with a low response value (low AUC = sensitivity to Node knockdown).

Table S5. Related to Figures 3&4. Drug screens. Mean AUCs across replicates from the single agent drug screen across 40 KRAS mutant lines are reported. Raw data, Bliss and Emax scores from the combination drug screen across 20 KRAS mutant lines are reported. Top hits from the combination drug screen are listed.

Table S6. Related to Figure 6. Causal network analysis. Mechanism gene expression footprints: gene expression footprints that predict the following mechanisms – STK11/LKB1 kinase activity, PDK1 (PDPK1) protein abundance, KLF4 protein abundance, SNAI1 mRNA abundance, TGFBR1 kinase activity, SMO catalytic activity. Relationship between mechanisms and KRAS/RSK-dependence: statistics on the relationship between mechanisms and KRAS/RSK AUC scores, including Pearson correlation and FDR between mechanism and KRAS/RSK AUC, Elastic Net Effect score for mechanism and KRAS/RSK AUC, Elastic Net Frequency score for mechanism and KRAS/RSK AUC. Evidence for edges between mechanisms: PubMed IDs of the studies demonstrating a relationship (i.e. edge) between two mechanisms used in the model.

Table S7. Related to Figure 6. RAS pathway networks. Correlations between phenotypic outputs of Node 1 and Node 2 in all cell lines. Average correlations of KRAS-, RSK- and RAL-type KRAS mutant lines are aggregated.

Supplemental Information

Supplemental Experimental Procedures

Sensor assay. The RasT2 RNAi Sensor assay was carried out as previously described (Fellmann et al., 2011; Pelossof et al., 2017; Yuan et al., 2014). The RasT2 set includes 83 human and 83 mouse RAS pathway genes (65 shRNAs/gene). To assess assay performance, several hundred previously tested shRNAs were included. This set comprised 6 genes (human *MYC* and mouse *Myc*, *Mcl1*, *Bcl2*, *Kras* and *Trp53*) that had been TILED as part of the original Sensor assay (Fellmann et al., 2011). Mouse *Hras* had also been TILED before, but the NCBI annotated transcripts have changed significantly, altering the sequence space for shRNA design. All 27 reference control shRNAs (spotted 2X on the chip) had been tested in multiple previous Sensor assay rounds.

siREN data processing to derive AUCs. FACSDiva files (.fcs) were converted to tab delimited (.txt) files using Cytobank(Kotecha et al., 2010). Raw.txt files were processed one line (i.e. one cell) at a time, and single cell data were binned into 75 bins based on EGFP-positivity. Bins with sparse cells (low standard deviation across cell lines) were removed and then the dataset was re-binned into 75 bins based on the trimmed data. The data were then normalized to one of 4 negative control samples, choosing the sample most proximal in position on the 96-well plate to account for drift in signal over time. Using the normalized data, AUCs for each node were derived based on a 5-bin moving window strategy. AUCs were computed using the R package MESS. Briefly, the areas underneath curves that spanned 5 consecutive bins were identified by scanning through all 75 bins. These "core" AUCs in either the dropout or enrichment ranges (negative or positive AUCs, respectively), were then expanded on both sides one bin at a time until the maximal absolute values were obtained for both dropout and enrichment AUCs. The same procedure was repeated for every node per cell line and was reported as the low or high EGFP range AUC. Replicates were averaged after removal of samples that were technical failures, resulting in the final AUC metric used for downstream analysis.

Distance to Death (D2D). Using Gaussian mixture models of cell counts based on EGFP and PI signal, we detected whether node knockdown elicited a death response (see next paragraph for details). Typically, the PI-positive population will increase when a siRNA induces death, and the knockdown population (moderate/low EGFP) will decrease when a siRNA induces growth inhibition or death. However, the way these populations shift can vary from cell line to cell line. We thus did not bias our method with assumptions on the behavior of PI and EGFP signals and instead, compared each FACS result with an internal positive control, siDEATH (AllStarsDEATH, Qiagen - a toxic cocktail of siRNAs), which was applied to each cell line and reported a cell line-specific death response. Within each cell line, we used the populations of the four Gaussian models to create vectors of size 4 for each node, and we computed the norm-1 distance to the positive control, node 26 (siDEATH 5uM), to assess the extent to which the siRNA induced death in the cells. We normalized the result by dividing the result by the distance between node 4 (AllStars Negative Control, Qiagen – a non-targeting siRNA) and node 26. Accordingly, a small distance to death (D2D) value indicates high similarity between the observed node and node 26, and a value of one or greater indicates that the effect is not closer to siDEATH than the negative control, node 4. For this reason, D2D values were capped at one. Values were computed on replicates 1 and 2 separately. Good reproducibility was observed. For subsequent analyses like the Elastic Net and clustering of the cell lines, distance to death values were averaged between replicates.

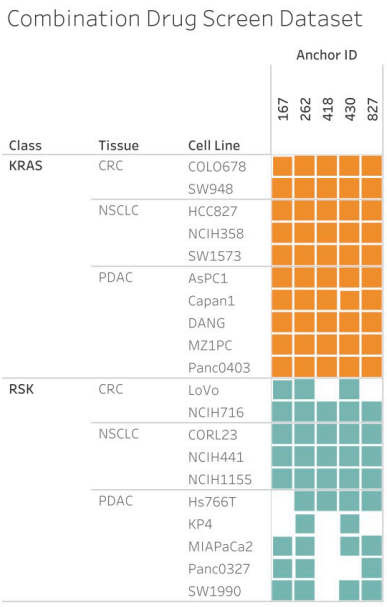
Gaussian mixture Fitting. To identify the different cell populations in the FACS data, we fit a Gaussian mixture on the joint distribution of the two channels, EGFP and PI (cell death), using the node 4 sample (siNegControl + siEGFP) for each cell line. The Gaussian mixture fitting was initialized by k-means clustering on a subsample of 3000 cells, and then fitted by expectation-maximization, using the package GaussianMixture.jl written in Julia (http://arxiv.org/abs/1411.1607, julialang.org) for efficiency (in this 2-dimensional case, the code ran a couple orders of magnitude faster than with the R package mixtools). The number of Gaussian components was fixed to 4 for all cases. We chose the node 4 (siNegControl + siEGFP) for fitting because it is likely to represent the most representative live cell populations: cells that received the siRNAs (low EGFP), cells that did not receive the siRNAs (high EGFP), cells that did not stably integrate the EGFP expression vector (no GFP) and a few dying cells (high PI). The parameters of four Gaussians were kept fixed for each cell line, and used to count the number of cells for each Gaussian, by summing the posterior probabilities of being emitted by the Gaussian, for all the other nodes. Therefore, importantly, input FACS data was NOT extensively gated, as the number of dead cells is indicative of the

siRNA effect. Additionally, this procedure is entirely algorithmic (Gaussian mixture fitting replaces manual gating by automatically defining the dead cell population) and does not necessitate manual human intervention, reducing human bias in the results and accelerating generation of the results (9981 FACS experiments were produced in this study).

Identification of the knockdown population and other FACS channels. To identify the knockdown population, we first matched the Gaussians between the two replicates. We computed the matching that minimizes the 4 pairwise distances between the Gaussian means. The knockdown population was identified as follow: first, we excluded the Gaussian with the highest mean PI value, i.e. the dead cells population. Then we reasoned that the knockdown population should increase when adding the siGFP alone (node 4), but not when adding siGFP and siDEATH together (node 26). Therefore, we selected the Gaussian with the highest cell numbers in node 4 (mean of the two replicates) compared to nodes 1 (no siRNA) and 26. The intensity of other channels (CellROX, CellTRACE, CellSIZE) was computed as the mean intensity of all cells in the FACS data, weighted by the probability of belonging to the knockdown population.

Drug response data curve fitting. The drug response data were processed plate by plate for each line based on Growth Inhibition: [(T-T0)/(C-T0)]*100, where C is the readout of DMSO controls, and T0 is readout for medium blank controls and T is the readout for each well of the drug treatment. The data were fitted using R package drc with extensive modifications of the procedure for exhaustive and iterative attempts of fitting function with changing starting values or parameters for the best fitting. For the reported data, we assume fixed upper limits and lower limits although more relaxed fitting were attempted as well. IC50s (or the minimum or maximum drug dose in the cases where the calculated IC50 was beyond the dosage range) and areas under the dose curve (AUC) were obtained. These metrics were log transformed and used in a two-sample t-test or Wilcoxon rank test to report the p-values for significance of the difference between RSK- and KRAS-type lines.

Drug combination screen. Cells were seeded in 384 well plates at an optimal density determined prior to screening for each cell line. In each plate 32 wells were not seeded with cells (blank wells). For each cell line 2 identical plates were seeded on a given date. At least 2 different seedings (on different days) were performed for each cell line and anchor drug with the goal to obtain biological replicates that passed quality control metrics. Both plates received 28 drugs (library drugs) at 9 different doses (Table S6) in a 2-fold dilution series. Forty-two wells did not receive library drugs and were used as controls. Fifty-eight wells at the periphery of the plates were seeded but not used (border wells). Combinations with the anchor drug was performed by adding the anchor drug to one of the 2 plates.



Drug Combination Screen Coverage. All cell lines were screened with all anchor drugs. In some instances (blank squares) the screen plates did not

All wells received the anchor drug including control wells. The second plate received DMSO (vehicle control) in all wells. After 5 days of incubation, CellTiter-Glo (Promega) was added to all wells (except border wells) and plates were read. Relative viability to the control wells was determined: For the plates that received DMSO, this corresponds to the single drug effect. For the plates that received the anchor drug, this corresponds to the effect over the anchor drug effect as the control wells in these plates received the anchor drug alone. In addition, for combination wells, the relative viability to no treatment was obtained by taking the fluorescence ratio in the anchor plates over the matched (same seed date) DMSO control wells in the DMSO plate. Synergy calculations were performed according to the Bliss independence hypothesis. Here we apply this hypothesis as follows: Vd xVa = Vd,a where Vd is the relative viability observed in the presence of the library Drug alone and Va the relative viability observed in the presence of the Anchor drug alone. The Bliss model predicts that Vd,a the product of these two viability ratios is the viability obtained for the combination of these drugs at the same doses than used as single agent. We obtained a synergy

estimate for each combination well by subtracting the observed relative effect in the combination plate (compared to Anchor alone) from the relative effect in the single agent plate (compared to DMSO alone). This allows us to compare effects of the drugs between plates rather than the raw fluorescence signal, minimizing technical noise in addition to making the liquid automation more efficient. Synergy for each combination on a given seed date is calculated by taking the second to maximum difference between relative viabilities across the 9 doses tested (2^{nd} to max excess over the Bliss model predicted effect across each dose tested). Replicate plates obtained at 2 different seed dates are treated independently and the final synergy score for a combination corresponds to the average of synergy score for all replicate plates that have passed QC. To determine the maximum viability effect (Emax) we use the ratio of the Anchor + Drug signal over the DMSO control wells (relative to untreated not relative to anchor alone) and take the minimum viability value observed across the 3 maximum doses for a given library drug. QC metrics are as follows, all criteria must be fullfilled: ratio of control wells / blank wells signal > 40 (signal over noise); Coefficient of variation of the control wells <20% (variability); Signal in the 2 minimum drug dose wells > 1.2-fold signal in the 2 maximum drug dose wells (correct dose response order). Response for highest two drug concentrations minus response for lowest two drug concentrations > .2 and < .8 (correct dose response range). For the abnormal drug response tests (order and range), we allow 2 out of 28 drugs to fail before failing the whole plate.

Causal mechanism analysis. To find potential causal mechanisms of siREN node knockdown across cell lines, we used a knowledge base from Selventa (selventa.com) of biomolecular relationships to compute the strength of 2616 mechanisms for each of the 91 cell lines we had expression data for(Martin et al., 2012; Thomson et al., 2015). We then investigated which of the 2616 mechanisms correlated with siREN AUC and D2D, using the elastic net (EN) regression, in the same way we did with mRNA expression. In addition to the EN analysis, we also assessed significance of univariate correlations between each mechanism strength score and each node AUC, and corrected for FDR with the Benjamini & Hochberg method(Benjamini and Hochberg, 1995) in the R programming language(RCoreTeam, 2015). The biological model we presented in this article shows nodes that were significant markers of sensitivity or resistance in either analysis (strictly positive frequency for the EN and FDR cutoff of 0.1 for the univariate analysis), limited to the two siREN nodes KRAS and RSK.

Quantitative PCR. Cells were treated with drug for 72h, and total RNA was isolated using TRIzol reagent (Thermo Fisher) and purified using RNeasy Plus kits (Qiagen) according to the manufacturers' recommendations. cDNA was synthesized using Advantage RT-for-PCR Kit (Clontech) and RT-PCR was performed using *Power* SYBR Green PCR Master Mix (Applied Biosystems). Primers are provided in Table S17.

Western blots. Cells were lysed in 1% NP-40 lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 50 mM NaF, 5 mM NaPPi, 1 mM PMSF, 0.5 mM DTT, 200 mM Na3VO4, Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher)). Lysates were cleared by centrifugation before analysis by SDS-PAGE. Antibodies are listed in Table S17.

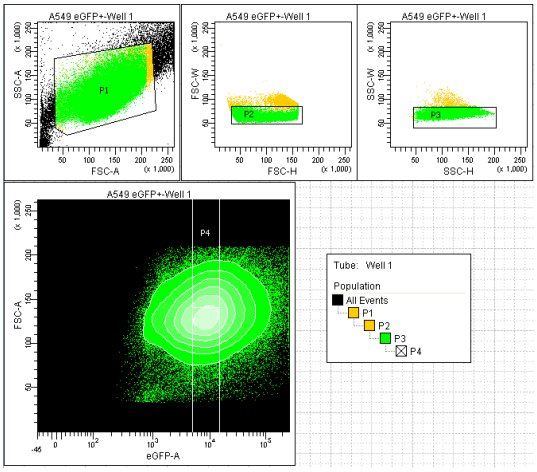
Lentiviral Transduction of cell lines
Cells were transduced with EGFP-expressing lentivirus at an MOI 0.3 in the presence of 8ug/mL polybrene.

Sorting for homogenous EGFP-positive populations

1) Gating scheme for single cells sorting:
    a. Ungated cells: FSC-A x SSC-A
        i. Draw P1 (live cells)
    b. Within P1: FSC-H x FSC-W
        i. Draw P2 (single cells)
    c. Within P2: SSC-H x SSC-W
        i. Draw P3 (true singlets)

2) Acquire P3 cells and sort for a tight P4 population of EGFP-positive cells.

3) Plate sorted cells into the pre-warmed media in the

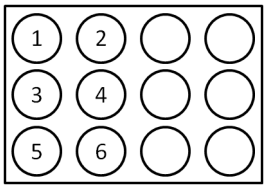


single well of the 6-well plate. Replace media 16-24h after sorting and expand the cells for experiments.

Transfection Optimization

1) Plate cells in 12-well plates and change the media to 900 µL of **P/S-free** complete media the day after seeding. Cells should be no more than 50% confluent.

2) Prepare the following stocks for transfection of siEGFP.



   a. Lipid solution: 6 µL RNAiMAX + 150 µL Opti-MEM
   b. siRNA solution: 0.4 µL of a 20 µM stock of siEGFP + 1.2 µL of a 20 µM stock of siNegCon + 200 µL Opti-MEM

3) Label six 1.5-mL tubes, one for each well, and add the appropriate volumes of reagents according to the table below:

Reagent	Well					
	0.25 lipid only	**2 lipid only**	**0.25**	**0.5**	**1**	**2**
	1	**2**	**3**	**4**	**5**	**6**
Lipid solution (µL)	6.25	50.0	6.25	12.5	25.0	50.0
siRNA solution (µL)	-	-	50.0	50.0	50.0	50.0
Opti-MEM (µL)	93.75	50.0	43.75	37.5	25.0	-
Total Volume (µL)	100.0	100.0	100.0	100.0	100.0	100.0

4) Briefly vortex and spin down each tube. Incubate at room temperature for 15 minutes.
5) Add the contents of each tube drop-wise to the corresponding well. Swirl plate gently. Incubate at 37˚C.
6) Allow cells to grow for 72 hours.
7) Label six cytometer-safe 5 mL polystyrene tubes with numbers 1 through 6. For each well, transfer the used media (with dead cells) to the appropriate tube.
8) Wash cells in each well with 1 mL PBS and harvest cells with 200 µL of Trypsin-EDTA. Transfer contents back to the appropriate 5 mL tube.
9) Secure tube caps and spin down cells at 100 x g for 5 minutes.
10) Wash cells with PBS.
11) With a pipette, remove as much of the PBS as possible without disturbing the pellet.
    - Cells can then be stained with PI and analyzed by flow cytometry immediately.
    - Alternatively, resuspend the cells in 350 µL - 2% paraformaldehyde. Secure tube caps and place tubes in 37˚C incubator for 10 minutes. Place samples at 4˚C until they can be analyzed by flow cytometry.
12) Measure the EGFP fluorescence of cells in gate P3. Estimate cell death by PI staining or the population of cells that are FSC-A (low); SSC-A (low-high) in fixed samples.
13) Determine the optimal volume of RNAiMAX to use for the siNODE transfections, based on the maximal eGFP knockdown and minimal cell death.

siREN Assay

siNODE Preparation

1) Each siRNA should be reconstituted at a 20uM. Refer to Table S1 for siRNA sequences.
2) Prepare the siRNA node mixes so each siRNA is added at 2nM per well. Final volume depends on node mix (see below) and includes 2nM of siEGFP and enough siRNA AllStars Negative Control siRNA (Qiagen) so the final siRNA payload per well is 8nM for nodes targeting 3 genes (or 12nM for the few nodes targeting 4-5 genes). Refer to Table S1 for node constituents.

Nodes:

NODE ID	Node Name	Vol (µl)	Volx3 (µl)
1	Lipid only	0	
2	Luci_1309	0.4	1.2
3	KRAS_911	0.4	1.2
4_1	NegCon	0.4	1.2
4_2	NegCon	0.4	1.2
5	KRAS_234	0.4	1.2
6	AKT	0.4	1.2
7	Apoptosis	0.4	1.2
8	Autophagy	0.4	1.2
9	Cdc42	0.4	1.2
10	Cell Cycle	0.4	1.2
11	Epigenetics	0.4	1.2
12	ERK	0.4	1.2
13	GAP	0.4	1.2
14	Glycolysis	0.6	1.8
15	Glutaminase	0.4	1.2
16	Hexokinase	0.4	1.2
17	Hypoxia	0.4	1.2
18	JNK	0.4	1.2
19	KSR	0.4	1.2
20	LDH	0.4	1.2
21	MEK	0.4	1.2
22	MSK	0.4	1.2
23	MTOR	0.4	1.2
24	NFkB_canonical	0.4	1.2

Tube	NODE	Vol (µl)	Volx3 (µl)
25	DEATH_1.0nM	0.4	1.2
26	DEATH_5.0nM	0.4	1.2
27	NFkB_non-canonical	0.4	1.2
28	NFkB	0.6	1.8
29	p38	0.5	1.5
30	PAK	0.4	1.2
31	PDK	0.4	1.2
32	PI3K	0.4	1.2
33	PLCE1	0.4	1.2
34	RAC	0.4	1.2
35	RAF	0.4	1.2
36	RAL_activation	0.4	1.2
37	RAL_effector	0.4	1.2
38	RAL	0.6	1.8
39	RAS	0.4	1.2
40	RAS_WT	0.4	1.2
41	RGL	0.4	1.2
42	RHO	0.4	1.2
43	RICTOR	0.4	1.2
44	ROCK	0.4	1.2
45	RSK	0.5	1.5
46	TCA Cycle	0.4	1.2
47	TIAM	0.4	1.2

siNODE Transfection

Due to timing of the assay, assaying up to 3 cell lines per day is recommended.

1) Seed 54 wells per cell line into 12-well plates (as shown below). 24h after seeding, replace media with 900 µl complete media that does not contain Pen-Strep.
2) For each cell line, prepare the reagents for the NODE transfection as follows:
    a. siRNA Solutions
        i. Label a 1.5 mL tube for each NODE ID and add 50 µl Opti-MEM.
        ii. To each tube add the volume listed in the chart above for the appropriate NODE.
    b. Lipid Solution
        i. Prepare 2.5 mL (55 wells x 50 µl) of RNAiMAX and Opti-MEM at the ratio determined by transfection efficiency for each cell line.

RNAiMAX/well	OptiMEM/well	RNAiMax/OptiMEM for 1 cell line (55wells)	RNAiMAX/OptiMEM for 3 cell lines
0.25 µl	49.75 µl	13.75 µl / 2.736 mL	41.25 µl / 8.208 mL
0.50 µl	49.50 µl	27.5 µl / 2.722 mL	82.5 µl / 8.166 mL
1.0 µl	49 µl	55 µl / 2.695 mL	165 µl / 8.085 mL
2.0 µl	48 µl	110 µl / 2.640 mL	330 µl / 7.92 mL

3) Add 50 µl of the Lipid Solution to siRNA solution for each node. Briefly vortex and spin down before incubating for 15 minutes at room temp.

4) Add 100 µl dropwise to each well according to the Node layout below. Swirl plate gently and incubate at 37°C.

Source Plate 1	1	2	3	4
A	1	2	3	4_1
B	5	6	7	8
C	9	10	11	12

Source Plate 2	1	2	3	4
A	13	14	15	16
B	17	18	19	20
C	21	22	23	24

Source Plate 3	1	2	3	4
A	25	26	4_2	27
B	28	29	30	31
C	32	33	34	35

Source Plate 4	1	2	3	4
A	36	37	38	39
B	40	41	42	43
C	44	45	46	47

Source Plate 5	1	2	3	4
A	No Dye Ctrl	Dye Ctrl		
B	No Dye Ctrl	Dye Ctrl		
C	No Dye Ctrl	Dye Ctrl		

CellTRACE Staining

Add 24 hours post-siNODE transfection

1) Warm CellTRACE (CellTrace Violet, ThermoFisher) reagents to room temp before beginning.
2) Add 20 µl DMSO to 1 vial of CellTRACE and mix to make a 5 mM stock solution.
3) Add 20 µl of the 5 mM stock to 40 mL of warm PBS to make the 2.5 µM CellTRACE working solution.
4) Aspirate the transfection media from the wells and add 400 µl of the 2.5 µM working solution. *Only add CellTRACE to the 3 'Dye Ctrl' wells on Plate 5.*
5) Incubate for 20 minutes at 37°C.
6) Aspirate the CellTRACE solution from each well and wash 1 time with complete media.
7) After wash is removed, add 1 mL fresh, warm complete media to each well.
8) Incubate at 37°C.

Media Addition

1) Add 150 µl new media to each well 72h post-siNODE transfection.

CellROX and PI staining

Add 96 hours post-siNode transfection and ~2.5 hrs prior to anticipated flow cytometry analysis start

1) Vortex and briefly spin down the CellROX (CellROX Deep Red Reagent, ThermoFisher) reagent.
2) Make a 750 µM solution of the CellROX by diluting 4.5µl of the 2.5 mM stock into 10.5µl of DMSO.
3) Add 15 µl of the 750 µM CellROX stock to 15 mL of warm fresh media to make a 750 nM working solution. *(These volumes are enough for 2 source plates.)*
4) Remove the old media containing dead cells from each well and transfer to a labeled 1.5mL tube. Store at 4°C until cells are ready to be harvested.
5) Using a repeater, add 400 µl of the CellROX working solution to every well. *Only add CellROX to the 3 'Dye Ctrl' wells on Plate 5.*
6) Incubate in the dark at 37°C for 1 hour.
7) When incubation is complete aspirate CellROX working solution and using a repeater rinse well with ~500 µl PBS.

Harvest Source Plates 1,2 and 5; submit for Flow analysis and then initiate harvest of Source Plates 3 and 4 for Flow analysis.
Note: Split the 'Dye and No Dye Ctrl' wells and submit in wells E1 and E2 on the first flow submission (source plates 1 and 2) for cytometer set-up

8) Using a repeater pipet, add 200 µl 0.25% trypsin and incubate at 37°C for 5-10 minutes.
9) When all cells have released, use a repeater to add 400 µl complete media.
10) Rinse the cells with 600µL from the edges of the plate and pipet up and down 4-5 times to re-suspend the cells.
11) Transfer cells to corresponding labeled 1.5mL tubes that contain the dead cells and media that was previously collected and stored at 4°C (step 4 in this section).
12) When all wells have been harvested centrifuge at 800 rpm, 4°C for 5 minutes.
13) Prepare the 3 µM PI working solution by adding 20 µl of the 1 mg/mL PI stock solution to 10 mL of PBS.
14) Aspirate the media gently taking care not to dislodge pellet.
15) Re-suspend the pellet in 250 µl 3 µM PI working solution. *Do not add PI stain to the 'No Dye Ctrl' sample, re-suspend in PBS only.*
16) Add 125 µl of each cell suspension to the 96-well plate in duplicate according to plate layout.
17) Load the Cell Dye controls into E1 and E2 with the plate 1 samples when submitting for flow.

96-well layout for Flow Cytometry Analysis submission (per cell line)

		1	2	3	4	5	6	7	8	9	10	11	12
First Flow Submission (Source Plates 1 & 2)	A	1_1_001 Lipid	2_1_002 Luci	3_1_003 KRAS_911	4_1.1_004 NC	5_1_005 KRAS	6_1_006 AKT	7_1_007 Apop	8_1_008 Autophagy	9_1_009 Cdc42	10_1_010 CellCycle	11_1_011 Epigenetics	12_1_012 ERK
	B	13_1_013 GAP	14_1_014 Glycolysis	15_1_015 GLS	16_1_016 HK	17_1_017 Hypoxia	18_1_018 JNK	19_1_019 KSR	20_1_020 LDH	21_1_021 MEK	22_1_022 MSK	23_1_023 MTOR	24_1_024 NFkB_Can
	C	1_2_025 Lipid	2_2_026 Luci	3_2_027 KRAS_911	4_1.2_028 NC	5_2_029 KRAS	6_2_030 AKT	7_2_031 Apop	8_2_032 Autophagy	9_2_033 Cdc42	10_2_034 Cell Cycle	11_2_035 Epigenetics	12_2_036 ERK
	D	13_2_037 GAP	14_2_038 Glycolysis	15_2_039 GLS	16_2_040 HK	17_2_041 Hypoxia	18_2_042 JNK	19_2_043 KSR	20_2_044 LDH	21_2_045 MEK	22_2_046 MSK	23_2_047 MTOR	24_2_048 NFkB_Can
Second Flow Submission (Source Plates 3 & 4)	E	25_1_049 DEATH_1	26_1_050 DEATH_5	4_2.1_051 NC	27_1_052 NFkB_NC	28_1_053 NFkB	29_1_054 p38	30_1_055 PAK	31_1_056 PDK	32_1_057 PI3K	33_1_058 PLCE	34_1_059 RAC	35_1_060 RAF
	F	36_1_061 RAL_acti	37_1_062 RAL_eff	38_1_063 RAL	39_1_064 RAS	40_1_065 RAS_wt	41_1_066 RGL	42_1_067 RHO	43_1_068 RICTOR	44_1_069 ROCK	45_1_070 RSK	46_1_071 TCA Cycle	47_1_072 TIAM
	G	25_2_073 DEATH_1	26_2_074 DEATH_5	4_2.2_075 NC	27_2_076 NFkB_NC	28_2_077 NFkB	29_2_078 p38	30_2_079 PAK	31_2_080 PDK	32_2_081 PI3K	33_2_082 PLCE	34_2_083 RAC	35_2_084 RAF
	H	36_2_085 RAL_acti	37_2_086 RAL_eff	38_2_087 RAL	39_2_088 RAS	40_2_089 RAS_wt	41_2_090 RGL	42_2_091 RHO	43_2_092 RICTOR	44_2_093 ROCK	45_2_094 RSK	46_2_095 TCA Cycle	47_2_096 TIAM

Supplemental References

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