**Supplementary Methods**

**Chuang et al. “Altered mitochondria functionality characterizes a metastatic cell state in**

**lung cancer and creates an exploitable vulnerability”**

**Mice**

*KrasLSL-G12D*, *Trp53flox* and *Rosa26LSL-tdTomato* mice have been described previously (1, 2). Tumors were initiated by intratracheal infection of mice with lentiviral vectors expressing Cre-recombinase as previously described (3). All animal studies and procedures were performed according to and approved by the Stanford Institute of Medicine Animal Care and Use Committee. Animals of both sexes were used.

**Cell lines**

The 368T1 and 394T4 cell lines were generated from primary lung tumors, the 238N1 and 482N1 cell lines were generated from lymph node metastases, the 404M1, 2691M1 cell lines were generated from distant organ metastasis, the 889PF from disseminated cancer cells within the pleural cavity, and the 579DLN cell line was generated from a distant lymph node metastasis of lung-adenocarcinoma-bearing *KrasLSL-G12D/+;Trp53flox/flox* (*KrasG12D;Trp53KO*) *KrasLSL-G12D/+;Trp53flox/flox;Rosa26LSL-Tomato/+* (*KrasG12D;Trp53KO;R26Tomato*) mice (2, 4). All cell lines were regularly (~ monthly) confirmed to be mycoplasma negative. 293T cell lines were obtained from ATCC (ATCC Cat# CRL-3216, RRID:CVCL\_0063) and identity was regularly confirmed by STR analysis. All cell lines were passaged at least once after thawing before use in subsequent experiments. The average time in culture was 4 weeks (range between 2 and 6 weeks), then a new vial was thawed.

**Cell line RNA-sequencing library preparation and analysis**

Total RNA was isolated from 1x106 cells of each line (368T1, 394T4, 238N1, and 482N1) using the Qiagen RNeasy mini kit. For each sample, 1 μg of total RNA was used for library construction. The integrity and quality of RNA was assessed prior to library construction using an Agilent Bioanalyzer. RNA-sequencing libraries were prepared using the Illumina TruSeq RNA kit according to manufacturer’s instructions. High-throughput sequencing was performed on Illumina HiSeq. For analysis of the RNA-seq reads, we performed differential gene and transcript expression analysis using TopHat (TopHat, RRID:SCR\_013035) and Cufflinks (Cufflinks, RRID:SCR\_014597). RNA-seq reads were separately aligned to the mouse genome (mm9) and the aligned RNA-seq reads were assembled into transcripts. Annotated transcripts were obtained from the UCSC genome browser (http://genome.ucsc.edu, UCSC Genome Browser, RRID:SCR\_005780) and the Ensembl database (Ensembl, RRID:SCR\_002344). Transcript abundances were measured in Fragments Per Kilobase of exon per Million fragments mapped (FPKM). Finally, Cuffdiff (Cuffdiff, RRID:SCR\_001647) was used to define differential expression. The data have been deposited in NCBI's Gene Expression Omnibus ((GEO), RRID:SCR\_005012) and are accessible through GEO Series accession number GSE159169 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE159169).

**Genome-wide shRNA screen**

Lentiviral particles from the 100K mouse shRNA pool (obtained from Broad Institute: The RNAi Consortium (5)) were transduced at low MOI (0.3; each shRNA was stably integrated into an average of 600 cells) into 4 cell lines (368T1, 394T4, 238N1 and 482N1; 3 replicates per cell line). Virus transduction and cell line passaging were performed according to the method described by Chueng et al. (6) with minor modification. In brief, the virus and cell mixture was seeded into a 12-well plate at ∼2 mL per well, and cells were transduced by spinoculation at 1000 g for 2 hours with 12 µg/ml polybrene at 30 °C. After 24 hours, the 12 wells from each replicate transduction were pooled, and the combined cells were transferred into a 15-cm dish. Cells were selected with 4 µg/ml puromycin starting day 4 post-transduction. For all subsequent passages, 7.2 × 107 cells per replicate were carried over. The remaining cells for all passages were collected, resuspended in 1 mL of PBS, and stored at −20 °C for genomic DNA isolation. Passaging for each cell line was continued for at least 22 population doublings. Puromycin selection was maintained for the entire experiment.

To identify the shRNA sequences enriched in screened cells, genomic DNA was isolated from cells collected at each timepoint, and hairpin sequences were quantified using Illumina DNA sequencing (7).

**Statistical analysis of the screen**

We applied the RNAi Gene Enrichment Ranking (RIGER), Kolmogorov-Smirnov (KS), and Weighted Sum (WS) algorithms to evaluate candidate genes from the pooled shRNA screens. Kolmogorov-Smirnov (KS) is nonparametric and calculates gene scores from a collection of shRNA phenotype profiles, similar to the method described by Luo et al. (8). Weighted Sum (WS) algorithms takes the combined sum of the first and second best ranks for hairpins for each gene. The best ranking hairpin is given a weight of 0.25 and the second best ranking hairpin is given a weight of 0.75. The weighted values (WS score) are determined by the sum of the first and the second weighted ranks, and genes are ranked by this WS score (https://software.broadinstitute.org/GENE-E/extensions.html). We considered all three ranking approaches to generate a list of top 50 candidate genes (Supplemental Fig. S2A) whose knock-down was found to impact only the Met cell lines in the screen (238N1 and 482N1), or only the Non-Met cell lines (368T1 and 394T4), or each cell line individually. Heatmaps for each set of genes were generated using the heatmap.2 function from the R package gplots (V 3.0.3). Clustering (depicted in Figures 1C and D) was performed according to https://software.broadinstitute.org/GENE-E/doc.html. Each box represents the measure of global correlation between the entire dataset for one cell line versus another.

**Secondary screen**

A list of candidate genes was generated from the initial screen as described above. Additionally, control genes (12 “lethal to all” and 5 “advantageous to all”, as well as 32 inert genes) were chosen (Supplemental Fig. S2A). For the secondary validation screen, a pooled library of 512 TRC shRNA clones was produced by cherry picking these clones from the sequence-validated TRC shRNA library used in the first screen. Each gene was targeted by 2 shRNAs.

The viral packaging and collection procedure was established based on the manufacturer’s guidelines (https://portals.broadinstitute.org/gpp/public/resources/protocols). In brief, 293T cells were plated in ten 15cm dishes (Falcon/Corning, Tewksbury, MA, USA) at 80.000 cells per cm2 in DMEM (Gibco, Grand Island, NY, USA, Carlsbad, CA, USA) supplemented with 10% FCS (Invitrogen), 2 mM L-Glutamine (Invitrogen) and Penicillin-Streptomycin (Invitrogen). The next day, for each plate separately, a Packaging-Plus Mix was prepared consisting of 6 µg of shRNA library, 10 µg psMD2.G, 20 µg psPAX2 and 60 µL PLUS Reagent (Invitrogen) in a total volume of 1.3 ml OptiMEM (Invitrogen) and incubated for 15 minutes. In order to obtain a transfection mix, 1.4 ml of OptiMEM containing 90 µL of Lipofectamine (Invitrogen) was added to the Packaging-Plus Mix and incubated for another 15 minutes before adding dropwise to the plated 293T cells. The medium was changed 24 hours post-transfection. The medium from both the 48 hours and the 72 hours harvests after transfection was combined and filtered through a 0.45 µm PES filter (Nalge Nunc, Rochester, NY, USA). Lentivirus aliquots were stored at -80 °C until used for transduction of the selected cell lines.

To perform large-scale infections, 3.6x106 target cells (MOI 0.3; each shRNA was stably integrated into an average of 2000 cells) were transduced for each replicate. The virus-cell mixture was split across a 12-well plate at 2 ml per well. A spin infection was performed by centrifugation at 1000 g for 2 h at 30 °C. After 20 h, the 12 wells from each replicate infection were pooled, and the combined cells were transferred into a 15 cm dishes. Transduced cells were selected with 4 µg/ml puromycin from day 4 after infection. For all subsequent passages, 1.2×106 cells per replicate were carried over. The remaining cells for all passages were collected, resuspended in 1 mL of PBS, and stored at −20 °C for genomic DNA isolation. Passaging for each cell line was continued for at least 22 population doublings. Puromycin selection was maintained for the entire experiment.

To validate genes that were specifically required for the survival or growth *in vivo,* 3x106 puromycin-selected transduced cells (238N1, 482N1) were injected into back flanks of 3 NSG recipient mice. Mice were analyzed 4 weeks after transplantation. Tumors were disaggregated to single-cell suspensions by mincing with razor blades and treated with collagenase IV, dispase, and trypsin at 37oC for 30 minutes. Genomic DNA was isolated from cells collected from each tumor, and hairpin sequences were quantified using Illumina DNA sequencing as described above.

**Statistical analysis of secondary screen**

To normalize the read count of each hairpin, raw read counts were divided by total counts of each sample (per million). Hairpins with normalized count values < 10 for across all early time point samples were eliminated from downstream analyses. Changes in shRNA representation over time were calculated by average read counts of late time points minus early time points.

**GO Term and Gene Set Enrichment Analysis (GSEA)**

To discover pathways driving pan- or metastasis-specific lethality top candidate genes identified in the screen were mapped to their direct human ortholog using information provided in the Mouse Genome Database (9) at [www.informatics.jax.org](http://www.informatics.jax.org). For GSEA analysis (10) (SeqGSEA, RRID:SCR\_005724), a rank-ordered list of candidate genes was analyzed using GSEA v2.2.0 software available from the Broad Institute (http://www.broad.mit.edu/gsea) and GO term analysis from The Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/, DAVID, RRID:SCR\_001881). Only genes with a human homolog were used for this analysis.

**Tumor dissociation and cell sorting**

For cell sorting, primary tumors and metastases were dissociated using collagenase IV, dispase, and trypsin at 37 oC for 30 minutes. After dissociation, the samples were continually on ice, in contact with ice-cold solutions, and in the presence of 2 mM EDTA and 1 U/ml DNase to prevent aggregation. Cells were stained with antibodies to CD45 (BioLegend Cat# 103133, RRID:AB\_10899570), CD31 (BioLegend Cat# 102423, RRID:AB\_2562186), F4/80 (BioLegend Cat# 123131, RRID:AB\_10901171), and Ter119 (BioLegend Cat# 116233, RRID:AB\_10933426) to exclude hematopoietic and endothelial cells (defined in combination as Lineagenegative). To analyze mitochondria membrane potential, cells were stained with 200 nM Mitotracker deep red (Invitrogen) for 15 min at 37 °C. A fixed number of GFP-positive control cells was spiked into each sample for normalization to account for staining artifacts due to different cell numbers in the samples. DAPI was used to exclude dead cells. FACSAria™ sorters (BD Biosciences) were used for analysis. To analyze ATP levels, equal numbers of cells were analyzed with the reversed CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to manufacturer’s instructions, using an ATP standard curve for calibration.

**Cell line transplantation and analysis**

6- to 10-week-old NSG mice of similar weights, randomized for both male and female animals, were used for cell transplantation experiments. For the subcutane­ous injections, 5x104 889PF or 579DLN cells were injected into both flanks of NSG recipient mice. Treatment of mice was started 7 days after transplantation with either 100 mg/kg phenformin or vehicle by oral gavage or 9 days after transplantation with either 1mg/kg doxycycline or vehicle by intraperitoneal injection once daily. Mice were analyzed 3 or 4 weeks after transplantation, respectively. For the intravenous injections 579DLN cells were treated with either phenformin [200 μM] or vehicle for 48 hours *in vitro*. Then cells were trypsinized and 1x105 cells were injected intravenously into the lateral tail vein. Starting at the day of injection, mice were treated daily either with 100 mg/kg phenformin or with vehicle control by oral gavage. For the short-term intravenous experiment, 579DLN cells were treated with either phenformin [200 μM], doxycycline [15 µg/ml], FCCP [2 µM], or vehicle for 48 hours *in vitro*. Then cells were trypsinized and 5x105 cells were injected intravenously into the lateral tail vein of immunocompetent 129/Bl6 F1 mice. Mice were sacrificed either three or seven days after injection and lungs were harvested, weighed, and analyzed by fluorescence microscopy and flow cytometry. During analysis of the animals the performing scientist was blinded towards experimental groups. No statistical method was used to predetermine sample size. The Stanford Institute of Medicine Animal Care and Use Committee or the Landesamt für Natur, Umwelt- und Verbraucherschutz des Landes Nordrhein-Westfalen (LANUV) approved all animal studies and procedures, respectively.

**Statistics**

Graphs and statistics were generated using the GraphPad Prism software (GraphPad Prism, RRID:SCR\_002798). Significance, where indicated, was calculated using the Wilcoxon test for non-normally distributed data. Significance was determined as a p-value < 0.05. No statistical method was used to predetermine sample size.

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