Supplementary Materials for

Hypoxia as a Therapy for Mitochondrial Disease

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Materials and Methods:

1. **Genome-wide Cas9-mediated Knockout Screen**
   a. **Virus Production**

   The genome-scale CRISPR knockout (GeCKO) library v1 was generously provided by the Zhang Lab. Library details have previously been published *(10)*. For library lentivirus production, 1.2×10^7 cells were placed in each of 25, T225 flasks in 50ml of full DMEM (Life Technologies 11995) media (containing Pen/Strep, 10% FBS). 18h later, media in each flask was replaced with 13ml of DMEM (no Pen/Strep, 10% FBS) and 2h later, media was replaced with 20ml OptiMEM (Life Technologies 31985-070; no serum, no Pen/Strep). A transfection mastermix was made by combining individually prepared mastermix A (94ml of OptiMEM, 2.4ml of Lipofectamine 2000 (Life Technologies)) and mastermix B (94ml OptiMEM, 2.1ml of PLUS Reagent (Life Technologies), 240μg of pVSVg plasmid, 360μg of psPAX2 plasmid and 480μg of GeCKO plasmid library). Mastermixes A and B were combined for 20m. 8ml of the final mastermix was added to each T225 flask of HEK293T cells. After 6h, the media was changed to 30ml of DMEM media (Life Technologies 11995 w/ 1% BSA (Sigma)) and cells were incubated for 48-72h, before virus-containing supernatant was collected. Virus was concentrated by centrifugation for 2h at 24,000 rpm in a SW32Ti rotor. Virus was resuspended overnight at 4C and stored at -80C prior to use.

   b. **Knockout Screen**

   K562 cells were obtained from ATCC and maintained in full DMEM media (10% FBS, Pen/Strep). K562 cells were grown in 1L spinner flasks (Matrical) on magnetic stir plates (Bellco). Cells were always passaged before reaching confluency (1×10^6/ml) and subcultured at a concentration of 1×10^5/ml.

      i. **Virus Infection**

      2.5×10^8 K562 cells were resuspended to a concentration of 1.5×10^6 cells/ml. Polybrene (Sigma) was added to 120ml of the K562 cell suspension at a final concentration of 4μg/μl. 2ml of this cell suspension was placed in each well of 5, 12-well plates. 10ul of virus was added to each well for a target MOI of 0.3, ensuring that most cells incorporated 1 or 0 lentivirus particles. Plates were centrifuged for 2h at 1000g and placed in an incubator for 1h, after which media was aspirated. 2ml of full DMEM media (10% FBS, Pen/Strep) was placed in each well and cells were resuspended. 12h later, all wells were pooled into a spinner flask with 800ml of full DMEM media. A sample was taken for virus titration to ensure that the target MOI

was obtained. 24h after the infection, Puromycin (Invitrogen) was added at a final concentration of 2µg/ml to begin selection for infected cells. Two independent infections (Infection 1 and Infection 2) were performed to control for variability in library infection.

ii. Passaging

Infected cells were passaged before reaching $1x10^6$/ml and maintained in Puromycin-containing conditions for one week after infection. At this point, $7x10^7$ cells were pelleted and stored as pre-treatment (Early) samples for each infection replicate.

iii. Experimental Selection

After 1 week of Puromycin selection, cells from each infection replicate were transferred to experimental conditions of (a) untreated cells, (b) moderate disease and (c) severe disease. $7x10^7$ cells were pelleted and resuspended in media corresponding to each experimental condition. The untreated condition was defined as DMEM media (11965-092) with 1mM pyruvate (Invitrogen) added. The moderate disease condition was defined as DMEM media with 100nM antimycin (Sigma) and 1mM pyruvate. The severe disease condition was defined as DMEM media with 100nM antimycin, without pyruvate. All media also contained 200µM uridine (*12*).

iv. Passaging in Experimental Conditions

Cells were subcultured at $1x10^5$/ml and passaged before reaching $1x10^6$/ml. At each passage, $7x10^7$ cells were pelleted and stored for intermediate screen time points.

c. Library Prep

Sequencing libraries were prepared as previously described (*10*). Briefly, DNA was extracted using the Qiagen Blood and Cell Culture DNA Maxi Kit from $7x10^7$ cells per experimental condition, for each infection replicate. DNA was then purified using Micro Bio-Spin columns (BioRad 732-6224). 25 PCR reactions were performed using Herculase II Fusion DNA Polymerase (Agilent) to amplify the single guideRNAs (sgRNAs) from genomic DNA, at a minimum coverage of 450x per guideRNA. 30µl from the first pooled PCR samples were used as input for the second PCR reaction, allowing for attachment of barcodes and sequencing adapters. Barcode replicates were included for the moderate disease condition to ensure that PCR errors did not substantially affect results. The final PCR products were run on an agarose gel and the correct size PCR products were gel extracted and sequenced on an Illumina HiSeq 2500.

d. Analysis

i. Processing of sgRNA Reads

Custom Python and Matlab scripts were written for processing of sequencing reads. Reads were trimmed to remove barcodes and sequences corresponding to the GeCKO library backbone. A custom bowtie library was created for mapping between sgRNA sequences and guide/gene names. Bowtie alignment was performed, allowing for single mismatches. Finally, guide abundance was compiled for each experimental condition and a matrix mapping guide name to abundance for all samples was created.

ii. Identification of Enriched and Depleted sgRNAs

1. Cell Viability Screen

In order to identify genes which are essential to cell viability, guide abundance was first normalized to total number of reads per sample. The fold-enrichment was calculated for untreated samples (day 21 after experimental selection) relative to pre-treatment conditions (immediately before experimental selection), for both infection replicates. As infection replicates were very well correlated ($r^2 > 0.8$), the top 500 most depleted genes (by RIGER (*41*) analysis) were found for each infection replicate. The 500 most essential genes were determined for both infection replicates and compared to the known list of mitochondrial proteins, or MitoCarta to identify essential mitochondrial genes (*42, 13*). Genes without entrez id mappings were excluded ($< 3\%$ of list). RIGER output was generated for each infection replicate (untreated relative to pre-treated conditions) and used for Gene Set Enrichment Analysis (GSEA) (*43*).

2. Enrichment Screen

In order to identify gene knockouts which allow cells to cope with mitochondrial dysfunction, fold-enrichment was calculated for moderate or severe disease conditions relative to pre-treatment (early) conditions. The most enriched genes were then individually checked for their overall effect on cell viability (untreated relative to pre-treatment conditions). Alternatively, fold enrichment was also calculated for untreated vs. disease conditions. However, this form of analysis confounds genes which are enriched in disease conditions or selectively depleted in untreated conditions. The top hit was found using either approach.

iii. RIGER Analysis

RIGER analysis (*41*) was used to generate a summary statistic by combining information corresponding to all sgRNAs for a given

gene. sgRNAs were pre-ranked by fold-enrichment between two conditions. The Kolmogorov-Smirnov method was used with 1000 permutations. Gene scores were not adjusted for the number of sgRNAs corresponding to a given gene.

   iv. **GSEA Analysis**
   Output from RIGER analysis was used for gene set enrichment analysis (*43*). All curated gene sets were used during analysis in GseaPreranked mode. All other parameters were set to default values.

2. **Lentiviral Cas9 Vector**
   Individual sgRNAs targeting VHL from the GeCKO library were cloned into the lentiviral Cas9 vector and used for follow-up experiments. Additionally, "dummy" non-targeting sgRNAs were generated as controls. The following oligonucleotides were used for lentivirus sgRNA cloning:

   Oligo_1: 5' – CACCG < 20bp sgRNA sequence> -3'

   Oligo_2: 5' AAAC <complimentary 20bp sgRNA sequence> - C – 3'

Oligo Name	Oligo Seq (5' → 3')
Lenti_VHL_1_F	CACCGAGTACCTGGCAGTGTGATAT
Lenti_VHL_1_R	AAACATATCACACTGCCAGGTACTC
Lenti_VHL_2_F	CACCGCAGGTCGCTCTACGAAGATC
Lenti_VHL_2_R	AAACGATCTTCGTAGAGCGACCTGC
Lenti_VHL_3_F	CACCGCGCGCGTCGTGCTGCCCGTA
Lenti_VHL_3_R	AAACTACGGGCAGCACGACGCGCGC
Lenti_VHL_4_F	CACCGGTGCCATCTCTCAATGTTGA
Lenti_VHL_4_R	AAACTCAACATTGAGAGATGGCACC
Lenti_VHL_5_F	CACCGTGTCCGTCAACATTGAGAGA
Lenti_VHL_5_R	AAACTCTCTCAATGTTGACGGACAC
Lenti_DUMMY_1_F	CACCGACTTCCCACTTCTTAGGTTG
Lenti_DUMMY_1_R	AAACCAACCTAAGAAGTGGGAAGTC
Lenti_DUMMY_2_F	CACCGTGGAGTGCACGCGAAAAGAA
Lenti_DUMMY_2_R	AAACTTCTTTTCGCGTGCACTCCAC
Lenti_DUMMY_3_F	CACCGGGCGTTAATTAAACTGTTTT
Lenti_DUMMY_3_R	AAACAAAACAGTTTAATTAACGCCC

The lentiCRISPRv1 plasmid was digested with BsmBI, gel-purified and phosphorylated by T4 PNK. Above oligonucleotides were separately annealed for

each sgRNA construct. The annealed product was ligated into the lentiCRISPRv1 plasmid and transformed into Stbl3 competent cells.

3. **Growth Curves**

All growth curves were performed in either K562, HT29 or HEK293T cells (ATCC). All conditions were counted in duplicate wells for each growth curve time point. Each growth curve was performed in independent experimental set-ups a minimum of two times in each cell type. Representative growth curves are shown in Fig 2.

   a. **Lentivirus VHL Growth Curves**

   K562 cells were infected using the lentivirus CRISPR construct containing sgRNAs targeting VHL or dummy guides, following the same protocol as used in the original GeCKO screen. Puromycin selection was started the day following the infection. Cells were allowed to grow in Puromycin-selection conditions for at least one week, before beginning growth curves. The VHL-edited cells and dummy-guide infected cells were grown at a starting concentration of 2.5×10^4 or 5×10^4 cells/ml in 24-well plates. Cells were placed in DMEM media (Life Technologies 11995, 10% FBS, Pen/Strep) with (a) DMSO, (b) 100nM antimycin (Sigma), (c) 100nM oligomycin (Sigma) or (d) 1µM piericidin (Santa Cruz). All conditions for growth curves contained 1mM pyruvate and 200µM uridine, reflecting the moderate disease condition. Cells for each condition were counted in duplicate wells every 24h for at least 3 days.

   b. **FG-4592 Growth Curves**

   FG-4592 was purchased from MedChem Express (HY-13426) and Cayman Chemicals (CAS 808118-40-3). Drug stocks were made in DMSO at 150mM. Cells were pre-treated with 40-75uM FG-4592 for at least 24h before beginning growth curves. This allowed for the HIF transcriptional response to begin prior to treatment with respiratory chain inhibitors. All cells were resuspended at a concentration of 2.5×10^4/ml in 24-well plates and placed in (a) DMSO, (b) 100nM antimycin, (c) 100nM oligomycin or (d) 1µM piericidin. Cells were counted daily (HEK293T and K562) or every other day (HT-29). Cells in all conditions were counted in duplicate or triplicate wells. At least two independent growth curves were performed with independent cell stocks for each growth curve presented in Fig 2.

4. **Apoptosis Staining**

K562 cells were pre-treated with ~75µM FG-4592 or DMSO for ~48h. They were then exposed to vehicle or RC inhibitors (at same concentrations as in growth curve) for approximately 16h. Annexin-V positive cells were measured by flow

cytometry using the Annexin V/Dead Cell Apoptosis Kit (ThermoFisher Scientific).

5. **HIF1α Immunoblotting**

Cells were exposed to DMSO, 100nM antimycin or 100nM oligomycin, ± 50μM FG-4592, in 6-well plates. Plates were then placed in normoxic (21% O_2) or hypoxic (1% O_2) conditions for 12-18h. Air tanks corresponding to 1% O_2 (balance nitrogen) or 21% O_2 were purchased from Airgas. Cell culture plates were placed in hypoxic chambers (Billups-Rothenberg), sealed and flushed with air corresponding to different conditions (normoxia or hypoxia) for 7-10m. After overnight treatment, hypoxia chambers were opened and cells were collected using standard methods and placed in RIPA buffer (Boston BioProducts). As the normoxic half-life of HIF proteins is less than 5 minutes at room temperature, all samples and buffers were handled at 4C. 30μg of protein were loaded onto an 8% SDS-PAGE gel (Invitrogen). HIF1α was detected using Bethyl A300-286A and the loading control, β-tubulin was detected using CST 2128S.

6. **qPCR**

24h prior to RNA collection, 2.5×10^5 HEK293T or HT-29 cells were seeded per well in a 6-well plate in 3ml of DMEM (Life Technologies (11995), 10% FBS). Cells were incubated in 50μM FG-4592, 100nM antimycin, 1μM piericidin as indicated. Total RNA was extracted using an RNeasy Mini Kit (Invitrogen). cDNA, generated by Superscript III Reverse Transcriptase (Life Technologies), was combined with Taqman Fast Advanced Master Mix and assayed by quantitative PCR. All CT values for genes of interest were normalized against the HPRT gene (probe/control). The ratio (probe/control) was set to 1 for vehicle-treated cells. Oligonucleotide primer pairs for GLUT1, HK2, LDHA and PDK1 were purchased from Life Technologies.

7. **Lactate Measurements**

Cells were placed in 6-well plates at a concentration of 1×10^5/ml and pre-treated with 50μM FG-4592 or DMSO for at least 24h. After pre-treatment, media was replaced so that it contained either (a) DMSO, (b) 100nM antimycin or (c) 100nM oligomycin ± 50 μM FG-4592. Cells were incubated in given conditions for 8h. Media was then collected, spun down to remove cell debris and subsequently used for lactate measurements. Lactate concentrations were measured using the YSI 2900 Biochemistry Analyzer. Samples were run in duplicate or triplicate for each set of conditions. Each set of conditions was tested in at least two independent experiments. A representative experimental set is shown in Fig 3.

8. **Oxygen Consumption Measurements**

48h prior to measurement, cells were seeded at $3\text{-}4\times10^4$ per well in XF24 24-well cell culture microplates in 200µl of normal growth media (DMEM (Life Technologies, 11965), 3.7g/L $NaHCO_3$ and 10% FBS) and were incubated at 37C in a 5% CO_2 incubator. 12h after seeding, 800µL of normal growth medium containing either FG-4592 or DMSO was added to each well, resulting in final drug concentration of 50µM. 15m prior to input of cell plate in XF24 Extracellular Flux Analyzer, media was replaced with 850µL of assay medium -DMEM (US Biological, D9800), 25mM glucose, 1mM pyruvate, 15.9 mg/L phenol red, 10% FBS and 25mM HEPES-KOH, pH 7.4. Assay medium contained either 50µM FG-4592 or DMSO. Measurements were performed at consecutive intervals of mixing (2m), waiting (2m) and measurement (4m). Basal measurements were collected 4 times. OCR measurements normalized to cell number were averaged from three independent trials.

9. **Zebrafish FG-4592 Treatment and Reporter Fish Imaging**
Fish were raised and treated according to institutional protocols and regulations on an approved IACUC protocol, HMS 04626. Transgenic reporter embryos [Tg(*phd3::EGFP*)] and wild-type sibling controls were exposed to 2.5µM FG-4592 in 0.1% DMSO at 96hpf. Fluorescence was assessed *in vivo* using a Zeiss Discovery V.8 fluorescent stereoscope equipped with an AxioCam MRc digital camera.

10. **Zebrafish Antimycin Survival Curves**
Wild-type zebrafish embryos (TU strain) were raised to 96hpf, and then exposed to 2.5nM Antimycin ± 2.5µM FG-4592. Embryonic death was assessed over the next 72 hours.

11. **VHL -/- Zebrafish Survival Curves**
vhl+/- were incrossed, and resultant embryos were raised to 48hpf (since *vhl-/-* show lethality at later time points) and then exposed to 2.5nM antimycin. Fish were then scored as *vhl-/-* or WT and heterozygotes. Embryonic death was assessed over the next 24h.

12. **VHL in-situ hybridizations**
In situ hybridization for *glut1* and *ldha1a* were performed on zebrafish embryos fixed in 4% paraformaldehyde at 120hpf, after 24h of treatment with 2.5µM FG-4592. In situ hybridization was conducted using standard protocols (http://zfin.org/ZFIN/Methods/ThisseProtocol.html).

13. **Breedings and General Animal Care for Mouse Work**

The Palmiter lab at University of Washington generously provided us with *Ndufs4* +/- mice. We then expanded our colony and bred sufficient *Ndufs4* -/- and control (*Ndufs4* +/- and WT) mice for experiments included in this manuscript. Pups were weaned and genotyped at ~25d after birth. All cages were provided with food and gel, as well as water bottles. Food and gel were replaced three times a week and cages were changed once a week. Mice were humanely euthanized if they lost more than 20% of peak body weight. *Ndufs4* +/- and WT mice are indistinguishable in all assays we tested and were therefore combined to serve as control samples. All animals were cared for under the guidelines of Partners Healthcare. All the animal studies were approved by the Subcommittee on Research Animal Care of the Massachusetts General Hospital, Boston, MA.

14. **Chronic Hypoxic, Normoxic and Mild-Hyperoxic Exposures**
Wild type and *Ndufs4* KO mice were exposed to chronic hypoxia (11% O_2), normoxia (21% O_2) or mild hyperoxia (55% O_2) at ambient sea-level pressure. Mice were placed in 60 liter plexiglass chambers that were given a gas mixture of compressed air and 100% N_2, compressed air alone or compressed air and 100% O_2 (Airgas Inc.). The gas flow rates were measured and controlled with rotameters and valves. Oxygen concentrations were measured several times each day at the outlet of the chambers using an oxygen analyzer (MiniOx I Oxygen Analyzer, Ohio Medical Corporation) and the flow rates of air, nitrogen and oxygen were modified if necessary in order to obtain a stable oxygen concentration of 11% in the hypoxic chamber and 55% in the mild-hyperoxia chamber. Soda lime (Sodasorb, Smiths Medical) (approximately 250g), was placed on the floor of each chamber to scavenge carbon dioxide (CO_2) produced by the animals and replaced every 3 days. The CO_2 concentration in each chamber as well as the temperature and the humidity were monitored continuously using a dedicated infrared CO_2 analyzer, thermometer and humidity meter (Extech CO200 Monitor, Extech Instruments). The total flow of fresh gas flushing each chamber was adjusted between 5 and 10 L/min to maintain the chamber CO_2 level below 0.4% and the relative humidity between 30% and 70%. Mice were exposed to gas treatment continuously for 24 hours per day, 7 days a week. The chambers were briefly opened three times a week to weigh the mice, evaluate their neurological status, clean the cages and add water and food.

Due to the extremely long duration of the experiment, several accidental deaths were observed in the different conditions due to severe infection (retinal conjunctivitis), blunt trauma and accidental CO_2 accumulation. As each of these were isolated incidents and unrelated to the experimental question, these data were not incorporated into the manuscript.

15. **Blood and Tissue Collection**

Mice were anesthetized with an intraperitoneal (i.p.) injection of ketamine (120 mg kg^{-1}) and fentanyl (0.09 mg kg^{-1}). Following tracheostomy, rocuronium (1 mg kg^{-1}) was injected i.p. to induce muscle relaxation. Volume-controlled ventilation was provided at a respiratory rate of 90 breaths min-1, a tidal volume of 10 ml kg^{-1}, PEEP 1 cmH2O and inspired O2 fraction (FIO2) of 0.21 (Mini Vent 845; Harvard Apparatus). Blood was collected by cardiac puncture after opening the chest via median sternotomy and tissues were collected and immediately frozen in liquid nitrogen.

16. Rotarod Activity Measurements

A rotarod machine (Ugo Basile) was used to measure the ability of mice to stay on an accelerating, rotating rod. Rotarod parameters were as follows: acceleration of 5rpm/m and a maximum speed of 40rpm. On each measurement day, three trials were performed, with individual trials at least 10m apart to allow mice to recuperate. The median time on rotarod is reported. If mice used their body to grasp the rod (rather than walking on it) for more than 10s, this time was recorded as time of fall. Age of measurements is +/- 5 days for practical purposes without any age bias between groups.

17. Spontaneous Locomotor Activity Measurements

The open field instrument (Med Associates Inc.) was used to measure spontaneous locomotor activity. Mice were blindly chosen for a given day and placed in open field chambers for 1h. Spontaneous locomotor activity was measured based on beam breaks and recorded by the instrument. The traces shown in Fig. 5E are representative of a sick, *Ndufs4* -/- mouse exposed to 21% O$_2$ (age of such sickness varies slightly between mice) and age-matched KO and WT mice exposed to 11% O$_2$, and WT mice exposed to 21% O$_2$. Age of measurements is +/- 5 days for practical purposes without any age bias between groups.

18. Histology

Mice were anesthetized as during tissue collection (see above). The chest cavity was opened and a catheter was placed in the left ventricle. The whole body was perfused with ice cold PBS and then with 4% PFA. The brain was dissected out, stored overnight in 4% PFA and then placed in 30% sucrose (in PBS) for two days.

Formalin-perfused brains were sectioned parasagittally at the olfactory lobes. Two transverse sections of cerebellum and brainstem were also collected: a rostral section with subjacent pons, and a more caudal section with medulla oblongata. Immunohistochemistry was performed on adjacent tissue sections using an antibody recognizing the microglial marker Iba-1 (Wako; 2μg/ml) according to methods described elsewhere (*44*).

19. Complex I Activity Assay

Complex 1 Activity was measured in cerebellum tissue from mice. Tissue was homogenized in 1ml of ice-cold PBS using a Qiagen TissueLyser II. Approximately 50-100mg of tissue was used as input material for the Complex I Enzyme Activity Microplate Assay Kit (ab109721, Abcam). Absorbance was read at 450nm wavelength and recorded every 30s for 135 total measurements. Background signal was not subtracted in data shown.

20. Erythropoietin (Epo) Measurements

Mice were exposed to 21% O_2 or 8.5% O_2 for 6h. Blood was collected as described above in EDTA-containing tubes. Plasma was then used for detection of Epo using the Mouse Erythropoietin Quantikine ELISA Kit (EP00B, R&D Systems).

21. Plasma Metabolomics

Lactate and α-hydroxybutyrate were quantified in mouse plasma by spiking in each corresponding isotope labeled standard (CDN isotope). A series of standard solutions of metabolites at 7 different concentrations were prepared in a surrogate matrix buffer (PBS buffer with 30g/L human serum albumin). 30uL of the mouse plasma sample were combined with 20uL of isotope labeled internal standard, vortexed for 10s and spun down for 10s. Metabolite extractions were performed using 70% acetonitrile. A Q Exactive Plus Orbitrap Mass Spectrometer coupled to a Dionex UltiMate 3000 UHPLC system (Thermo Fisher Scientific) was used for LC-MS. The Xbridge amide HILIC column (2.1 X100 mm, 2.5 µM particle size, from Waters 186006091) was used to separate metabolites and MS was acquired under the negative ionization mode. The column was maintained at 27°C during runs. The mobile phase A was 20mM ammonium acetate, 0.25 % ammonium hydroxide pH adjusted to 9. The mobile phase B was 100% acetonitrile. The MS data acquisition was full scan mode in a range of 70–1000 m/z, with the resolution set at 140,000, the AGC target at 3E6, and the maximum injection time at 400 msec.
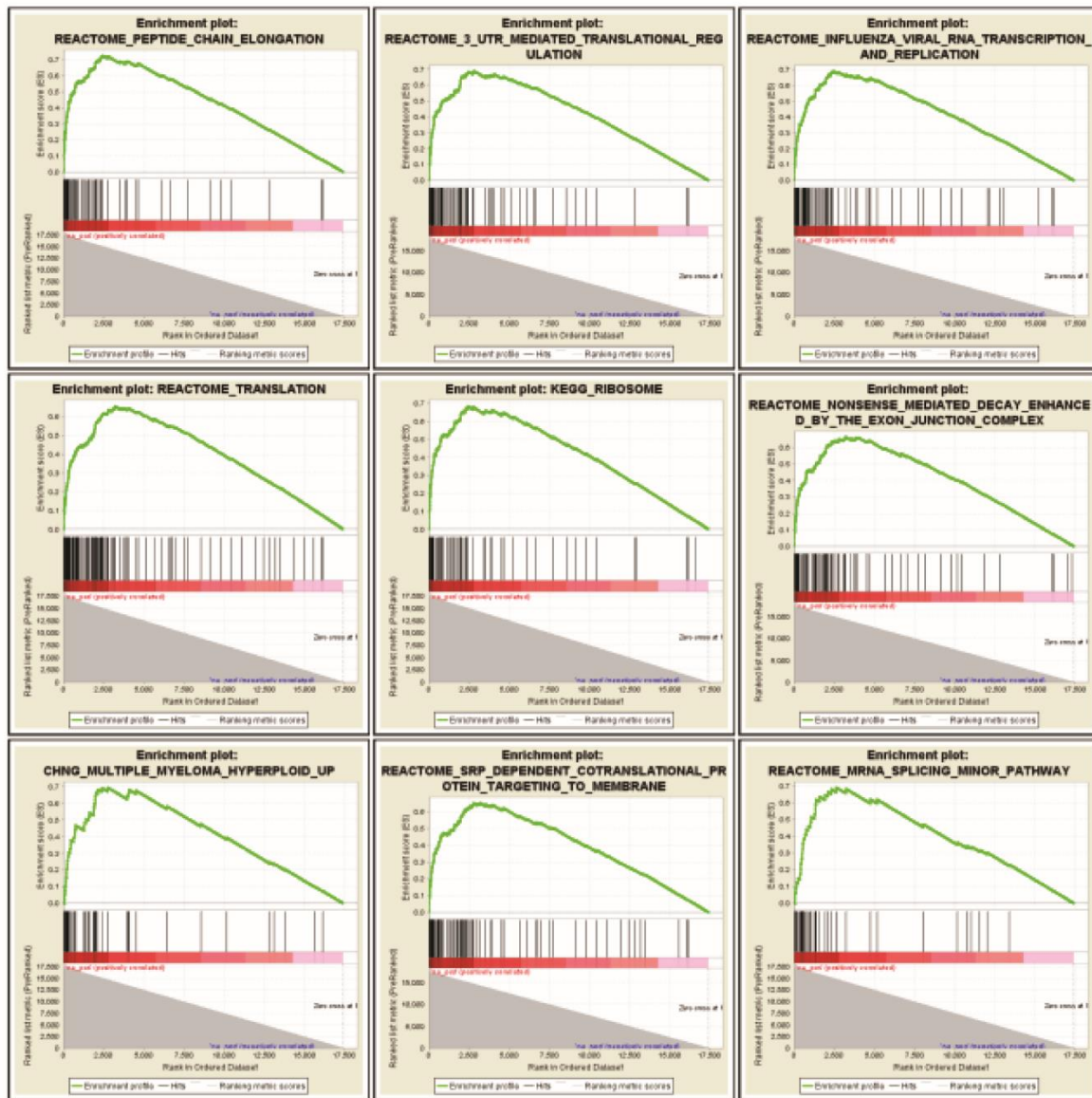
Figure S1. **Essential genes and gene categories are depleted in screen over time.**
Gene set enrichment analysis (GSEA) was performed using the depletion of sgRNAs
over time in untreated conditions (untreated relative to early (pre-treatment)). Shown are
GSEA plots for the curated gene sets that were the most depleted over three weeks of
growth. Each plot reflects a single gene category. Black lines reflect ranks of individual
genes within gene set. Most depleted gene sets correspond to essential cell functions.
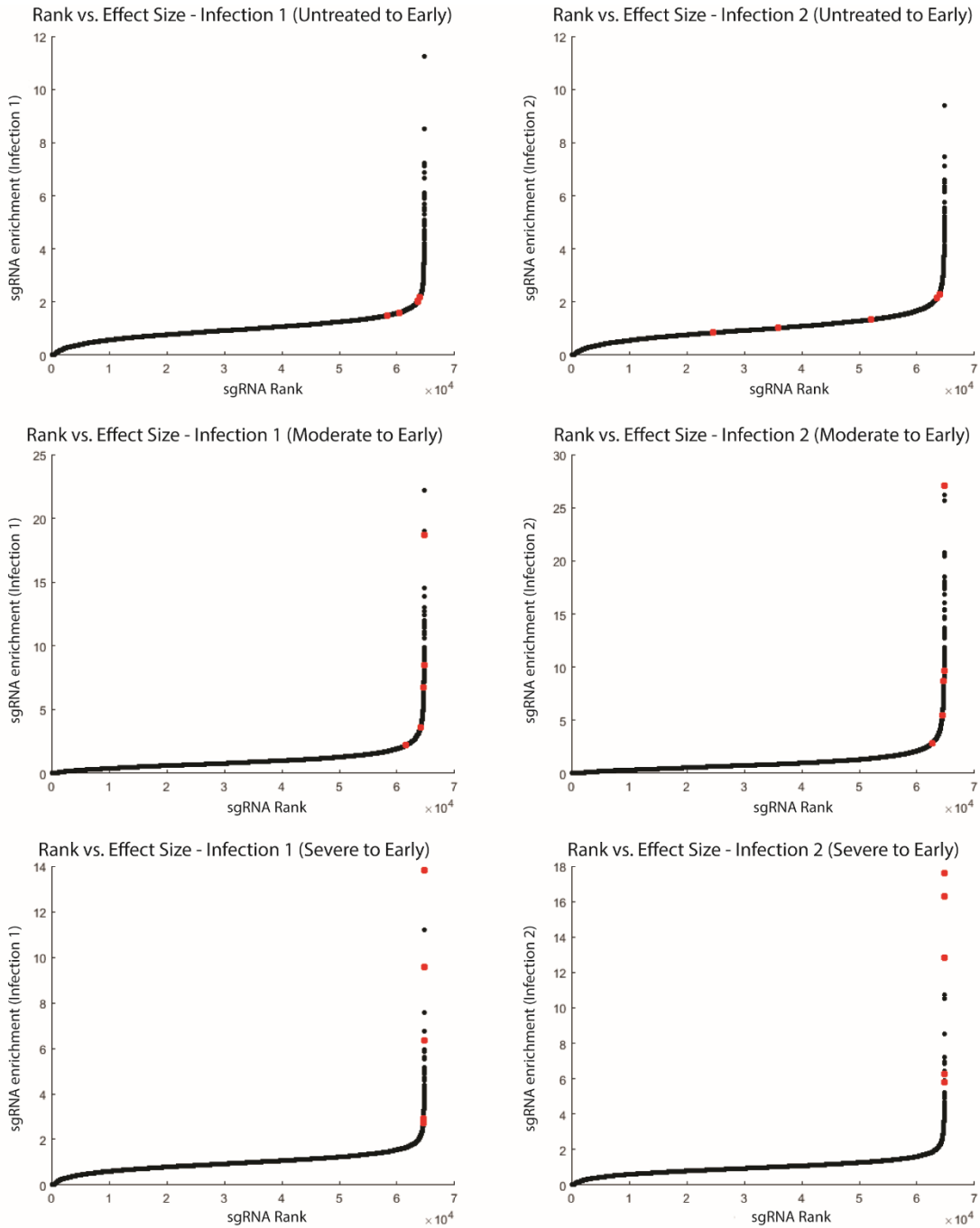
Figure S2. sgRNA enrichment vs. rank for all experimental conditions and infection replicates, relative to pre-treatment time point. sgRNA enrichment magnitude vs. sgRNA enrichment rank for each of ~65,000 sgRNAs included in the screen. sgRNAs corresponding to VHL are shown in red. Data is shown for both infection replicates for untreated vs. early conditions (top row), moderate disease vs. early conditions (middle row), and severe disease vs. early conditions (bottom row).
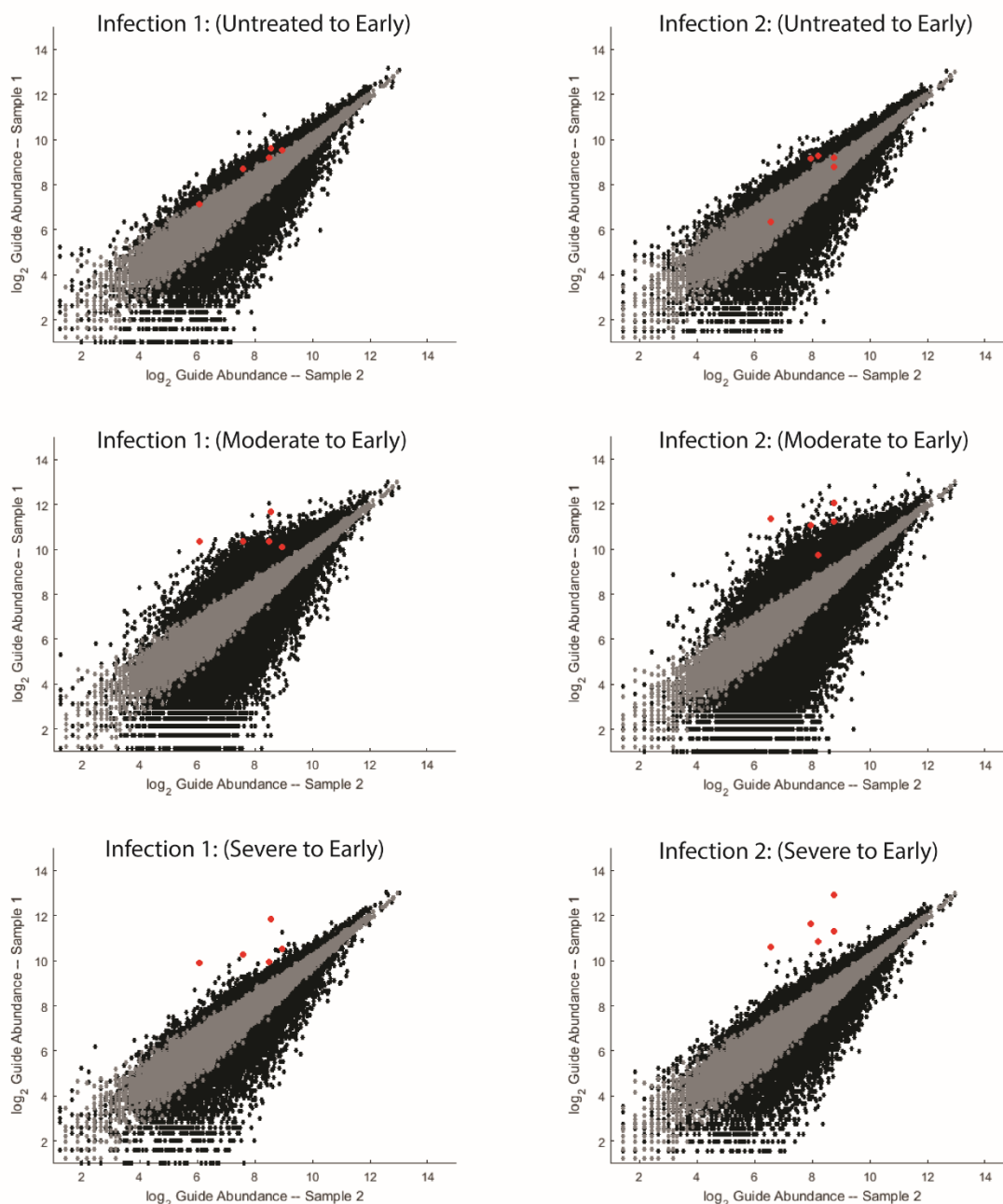
Figure S3. sgRNA abundance for all experimental conditions and infection replicates, relative to pre-treatment time point. sgRNA abundance in pre-treatment conditions (Infection 1 vs. Infection 2) shown in grey, reflecting experimental noise. Guide abundance in a given condition vs. early time point is shown in black, with VHL sgRNAs shown in red. Each dot represents an individual sgRNA. Data is shown for both infection replicates for untreated vs. early conditions (top row), moderate disease vs. early conditions (middle row), and severe disease vs. early conditions (bottom row).
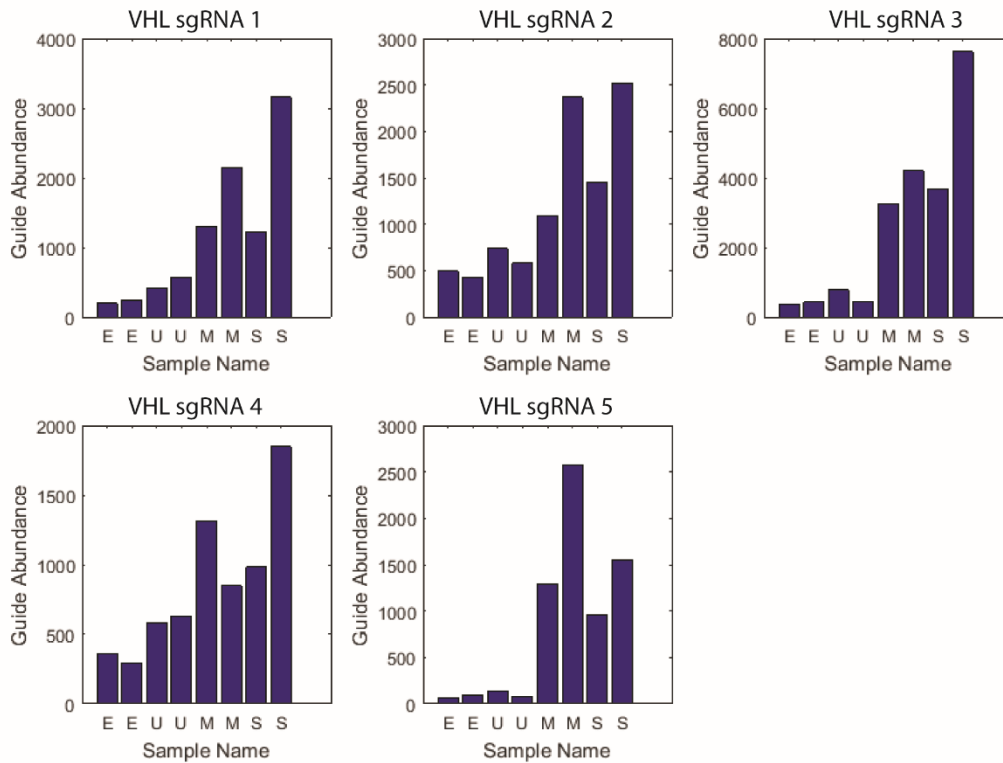
Figure S4. Abundance of sgRNAs targeting VHL across all samples. sgRNA abundance is shown for each of five, VHL sgRNAs. Data is shown for each individual infection replicate for the early time point, untreated conditions, moderate disease conditions and severe disease conditions. All sgRNA abundance values were normalized to total number of sequencing reads for a given condition.
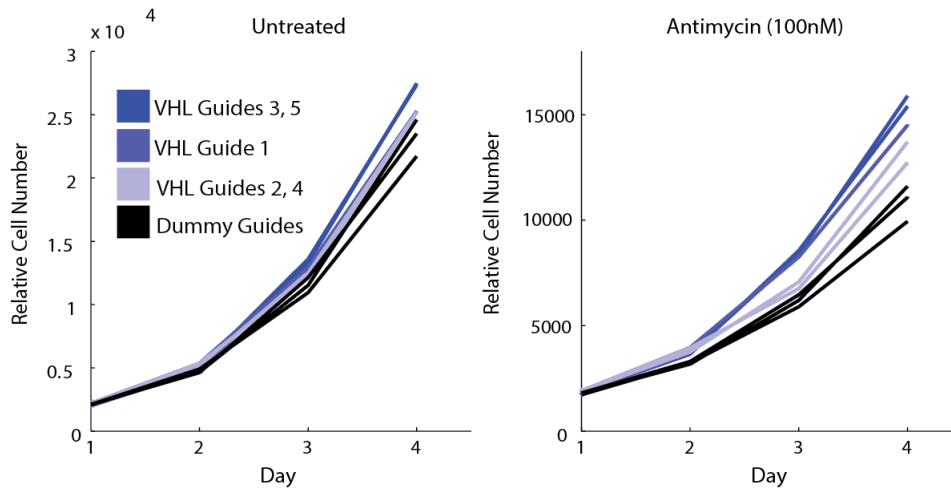
Figure S5. Rescue size of VHL sgRNAs, in presence of RC inhibition, is correlated with relative enrichment in screen. Growth curves for lentiviral VHL sgRNAs and dummy sgRNAs in untreated (left) or antimycin (right) conditions. sgRNAs are colored by their relative enrichment in the CRISPR screen. The strongest guides are shown in darker blue, and weaker guides are in light blue. Dummy guides are shown in black. All experiments were performed in duplicate wells. Growth curves are representative of at least two independent experiments.
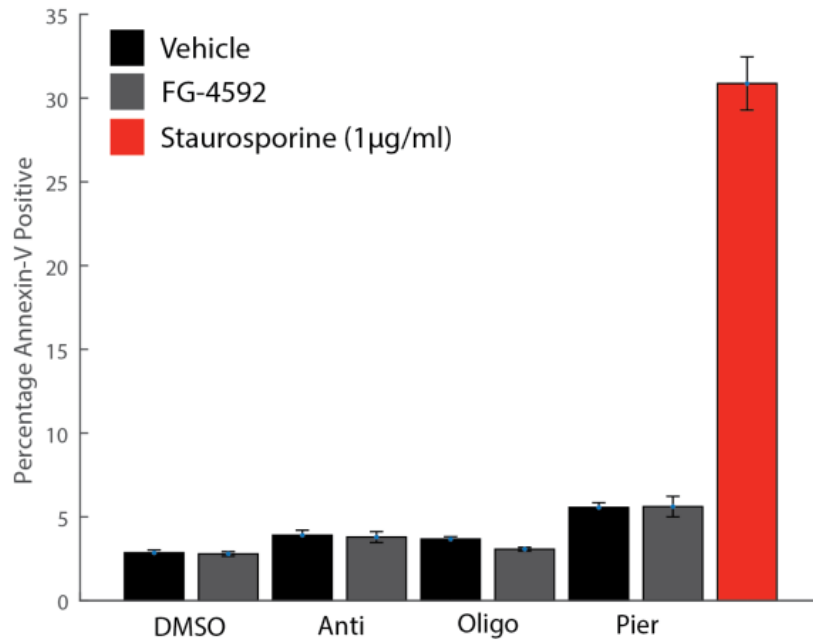
Figure S6. RC inhibitors do not substantially affect apoptosis levels in K562 cells.
K562 cells pre-treated with FG-4592 or DMSO for ~48h were then exposed to vehicle or
RC inhibitors for ~16h and stained for the apoptosis marker Annexin-V. Percentage of
Annexin-V positive cells were measured by flow cytometry. Staurosporine was used as a
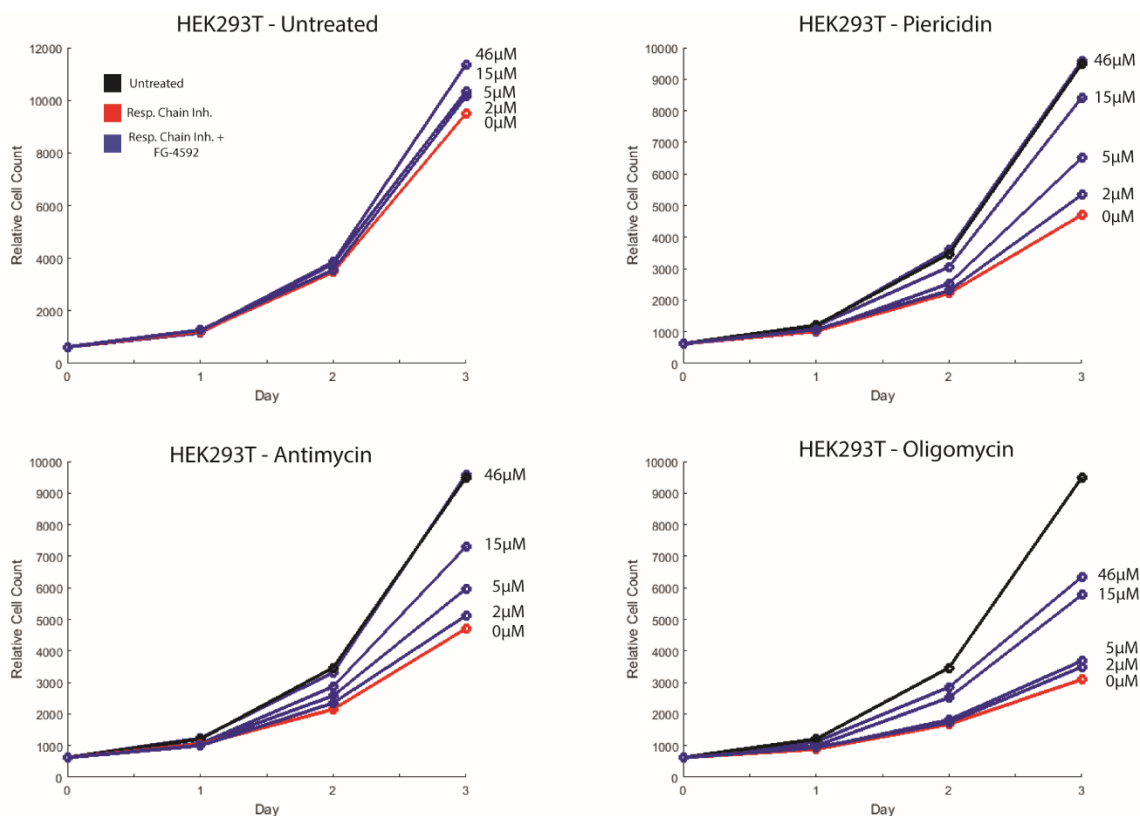positive control for inducing apoptosis.

Figure S7. FG-4592 causes a dose-dependent rescue of the growth defect caused by multiple forms of respiratory chain inhibition. Growth curves are shown for increasing concentrations of FG-4592 in untreated or RC inhibition conditions in HEK293T cells. Untreated growth curve is shown in black. Growth with RC inhibition, but without FG-4592 is shown in red. Increasing doses of FG-4592 shown in blue, reflecting increasing rescue during respiratory chain inhibition.
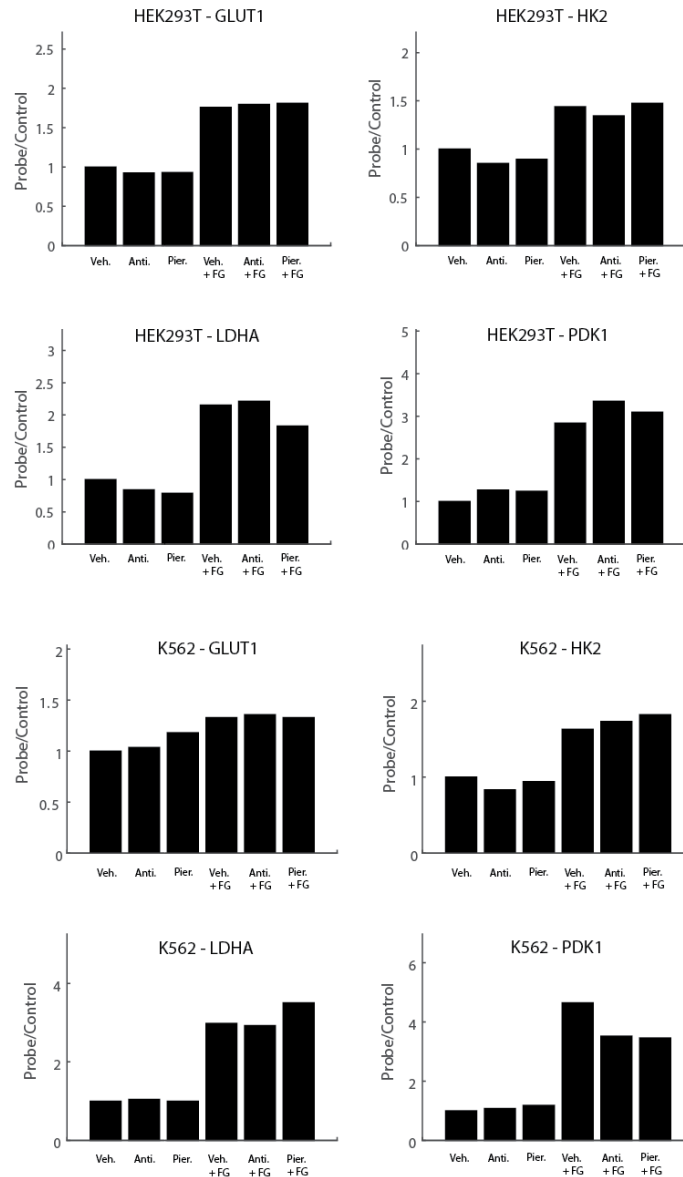
Figure S8. FG-4592 treatment activates transcription of HIF-responsive genes, whereas RC inhibition alone does not. Normalized transcript levels for the known HIF targets glucose transporter 1 (GLUT1), hexokinase 2 (HK2), lactate dehydrogenase A (LDHA) and pyruvate dehydrogenase kinase 1 (PDK1) +/- RC inhibition, +/- FG-4592. All data is averaged over three independent experiments (Mean ± S.E.) and normalized so vehicle-treated expression (probe/control) is set to 1. Data is shown for two additional cell types (K562 and HEK293T cells).

Condition	Mean	STE
WT (21% O_2)	56 pg/ml	+/- 17 pg/ml
WT (8.5% O_2)	2775 pg/ml	+/- 1070 pg/ml
KO (21% O_2)	77 pg/ml	+/- 37 pg/ml
KO (8.5% O_2)	2788 pg/ml	+/- 324 pg/ml

Figure S9. WT and *Ndufs4* KO mice are able to increase Erythropoietin levels after acute hypoxia exposure. 3 mice per group were exposed to 8.5% or 21% O_2 for 6h. Plasma erythropoietin levels were measured (Mean ± S.E.)
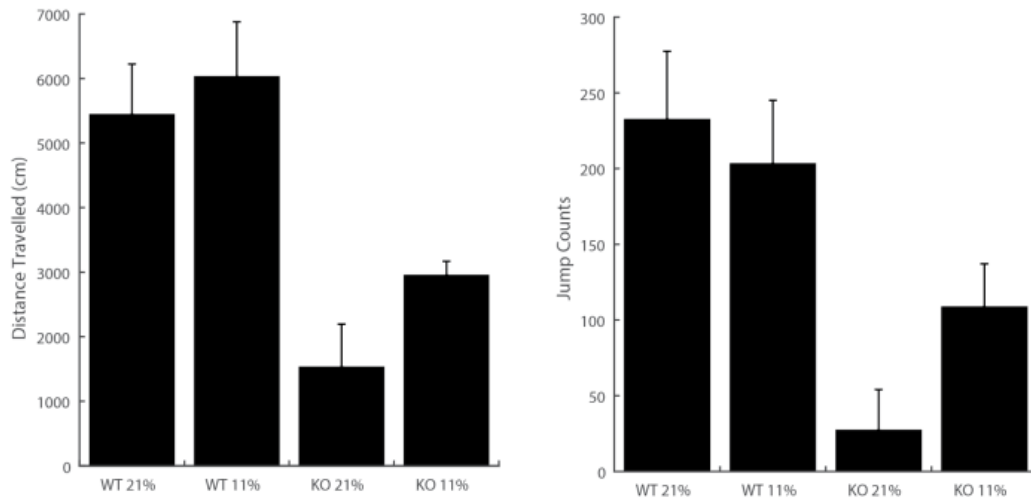
Figure S10. Spontaneous activity was measured in WT and *Ndufs4* mice exposed to normoxia or hypoxia. Distance travelled and jump counts within 1h are shown (Mean ± S.E.). n = 7, 5, 9, 9 for WT (21% O_2), WT (11% O_2), KO (21% O_2), KO (11% O_2) respectively.

Additional Data Table S1 (separate file)
Listed are the genes which affect cell viability the most in genome-scale Cas9-mediated knockout screen. RIGER ranking was used to determine the effect on cell viability by comparing untreated samples to early time point for both infection replicates. MitoCarta genes have a "1" in the second column. Data for both infection replicates shown as separate tabs.

Additional Data Table S2 (separate file)
RIGER output shown for comparing data from experimental conditions relative to pre-treatment (early) time point. Ranking is shown for all three conditions (untreated, moderate disease and severe disease), for both infection replicates as separate tabs.