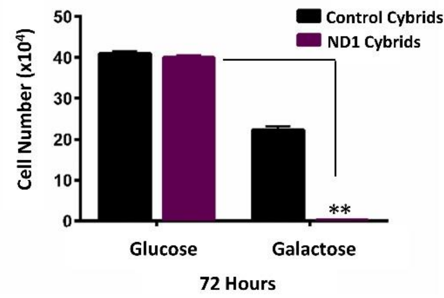
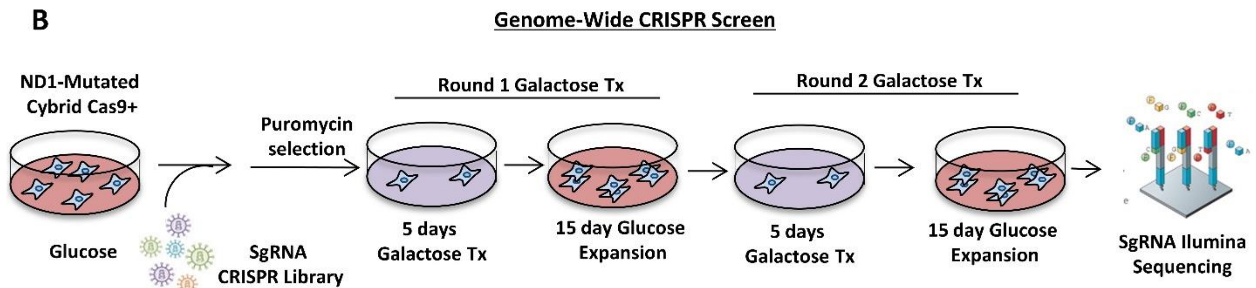
Figure S1

A



B



C

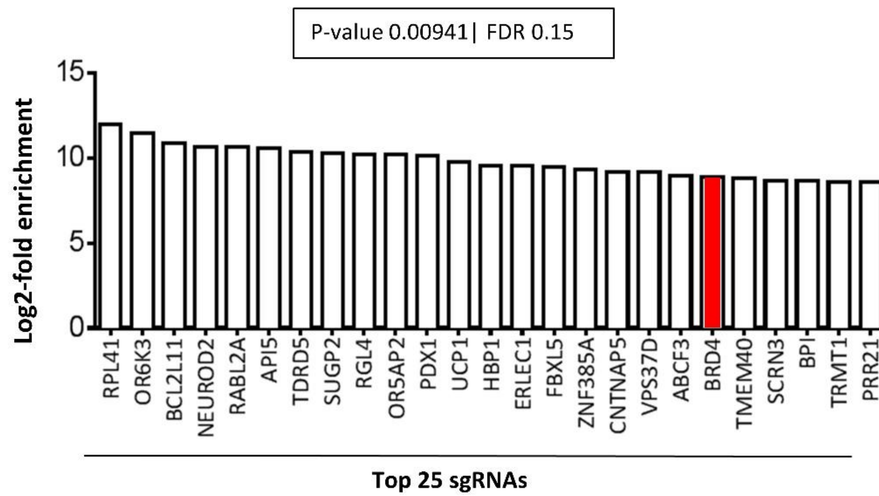


Figure S2

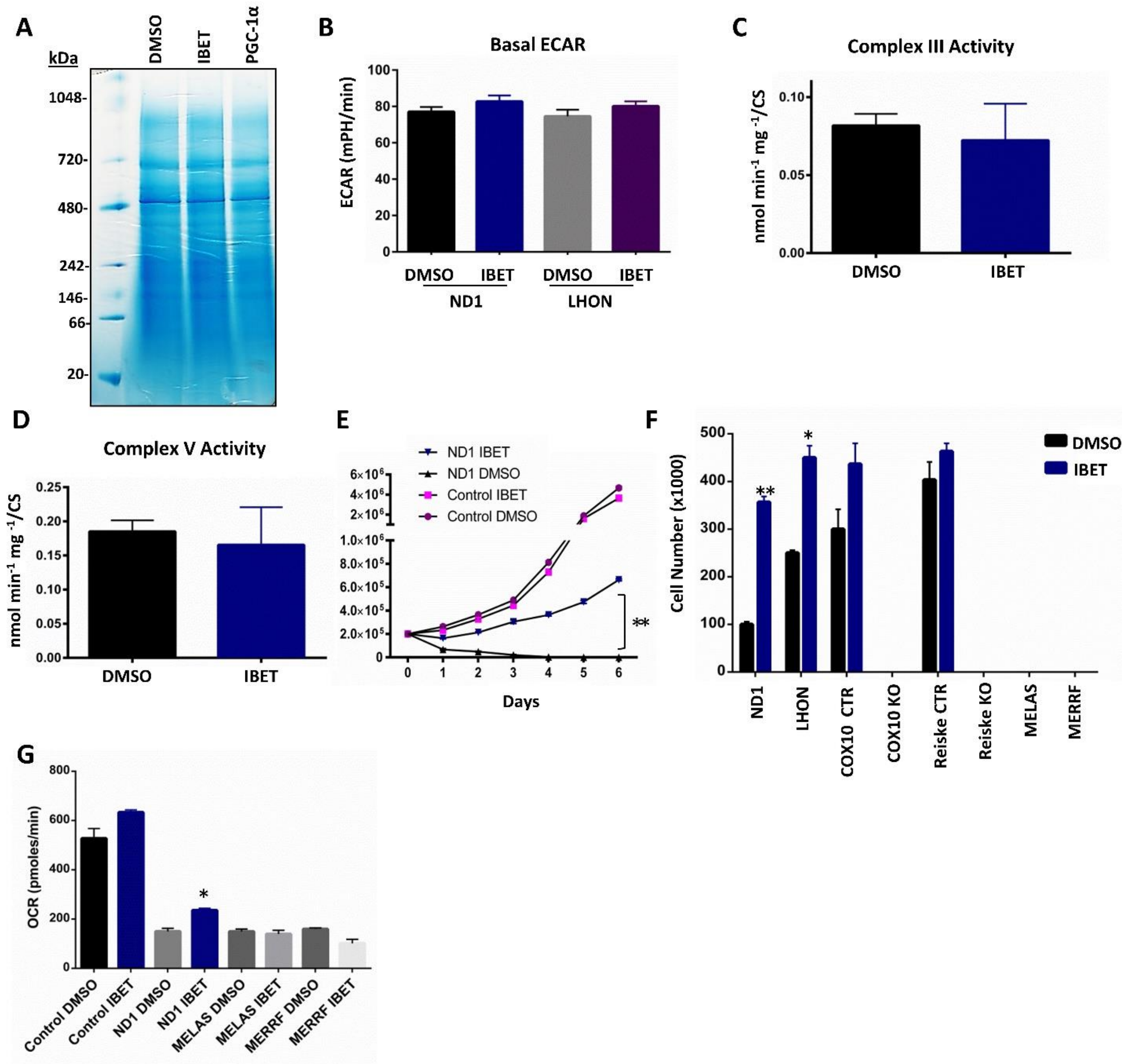
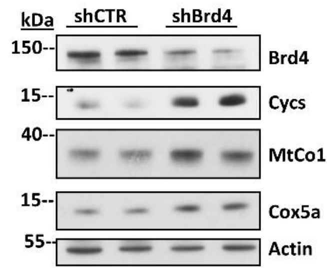
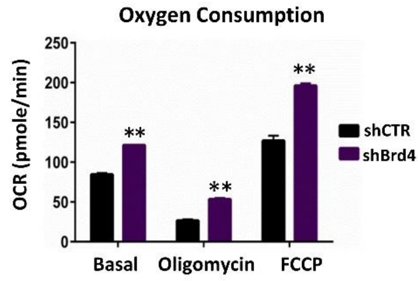
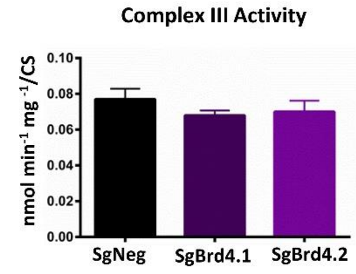


Figure S3







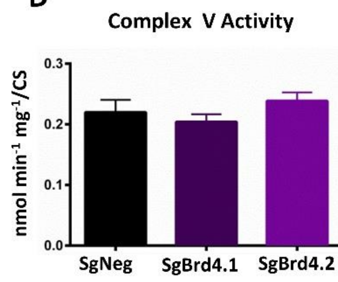


Figure S4

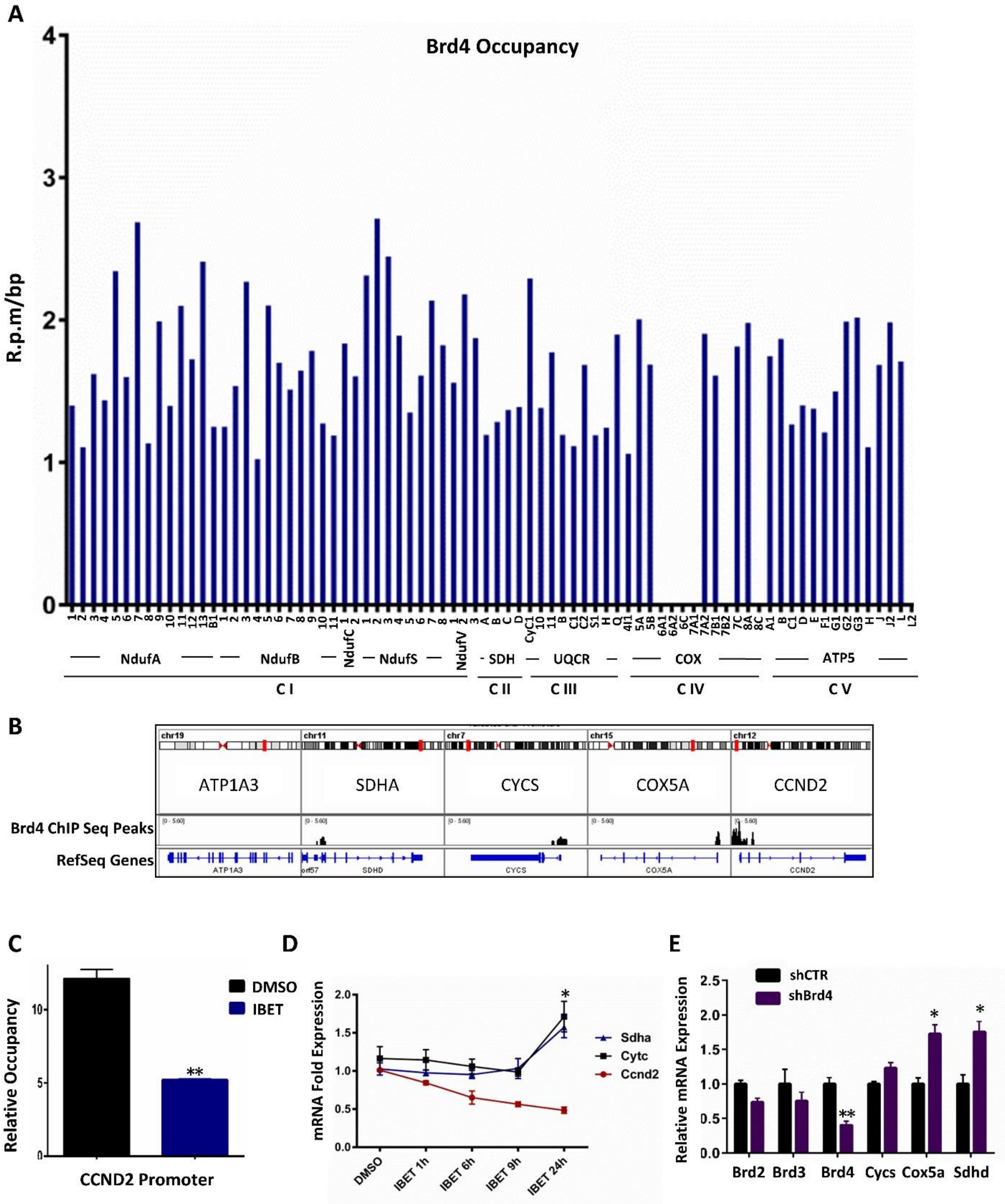
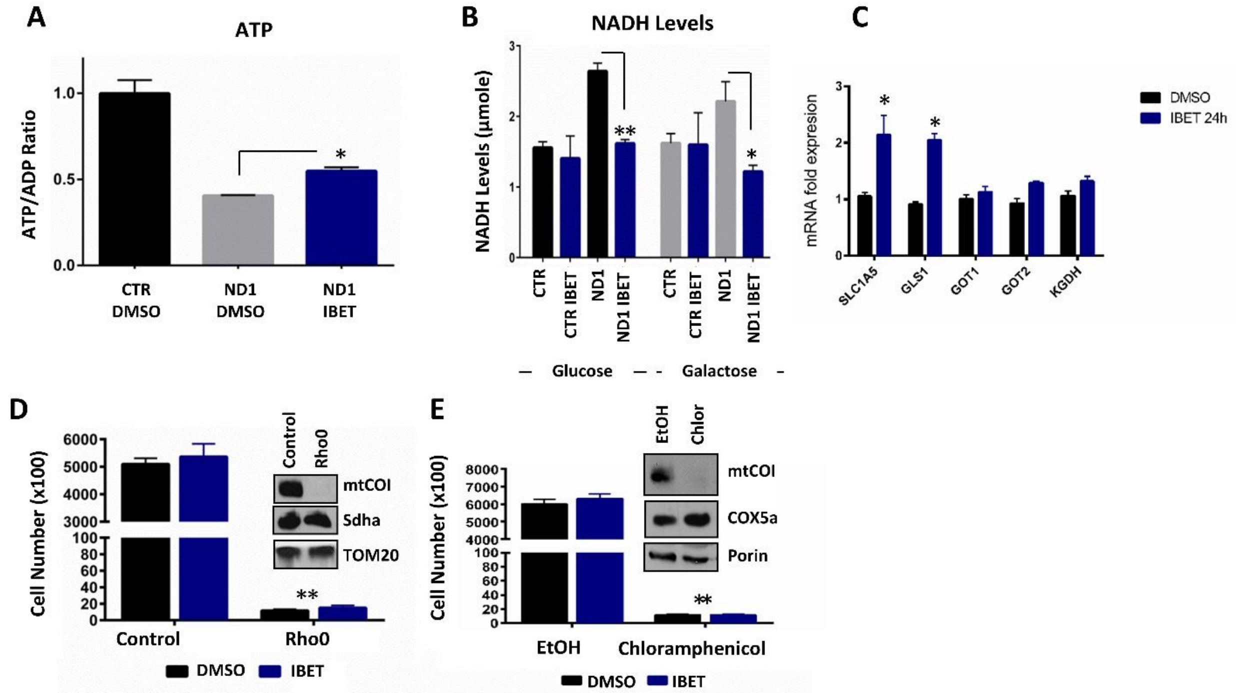


Figure S5



Supplemental Figure Legends

Figure S1. Related to Figure 1: Secondary CRISPR screen validation showed significant enrichment of Brd4 guides. A, ND1-mutant cybrids die within 72 hours with galactose treatment compared to control cybrids. Data are representative of the mean ± s.e.m., n=3. Asterisks denote $**p<0.01$ via Student's t-test. **B,** Workflow of CRISPR screen with second challenge of galactose treatment. **C,** Log2 fold enrichment of the 25 most significant guides after two rounds of galactose treatment.

Figure S2. Related to Figure 2 and 3: I-BET treatment rescues and promotes proliferation in CI-deficient cybrids under nutrient-restrictive conditions. A, Coomassie staining of blue native gel demonstrating equal loading in DMSO, I-BET, and adPGC-1α treated ND1-mutated cybrids **B**, I-BET 525762A does not affect the extracelluar acidification rates (ECAR) in ND1 and ND6 (LHON)-mutated cybrids. **C-D**, CIII (**C**) and CV (**D**) activities are not altered by I-BET treatment in ND1-mutated cybrids. **E**, Time-course-I-BET rescues and promotes proliferation of ND1-mutated cybrids when placed under galactose culture conditions. **F**, I-BET 525762A rescues CI (ND1 and ND6-mutated) cybrids under nutrient-restrictive galactose conditions. Rescue however was not observed for tRNA-mutated cybrids (MELAS and MERRF) or, CIII (Reiske) and CIV (Cox10)-deficient fibroblasts. G, Basal oxygen consumption is comparable between mutant cybrid cell lines. Data are representative of the mean ± s.e.m., n=3. Asterisks denote $*p<0.05$ or $**p<0.01$ via Student's t-test. All I-BET treatments are delivered at a final concentration of 0.9 μM

Figure S3. Related to Figure 4: Brd4 knock-down increases OXPHOS transcript and protein levels and enhances oxygen consumption in complex I-deficient cells.
A-B, shBrd4 ND1-mutated cybrids display increases in OXPHOS protein **(A)** and significantly enhances oxygen consumption in ND1-mutated cybrids **(B)**. **C-D**, Crispr-mediated ablation of Brd4 does not alter CIII (**C**) or CV (**D**) activity. Immunoblot is representative of at least 3 independent experiments. All other experiments represent the mean ± s.e.m., n=3. Asterisks denote $*p<0.05$ or $**p<0.01$ via Student's t-test delivered at a final concentration of 0.9 μM

Figure S4. Related to Figure 5: Brd4 is localized to OXPHOS promoters.
A, List of OXPHOS promoters (x-axis) with the average Brd4 occupancy ± 1000bp of the target gene's TSS plotted on the y-axis (represented as reads per million per base pair (R.P.M/bp). **B**, Representative OXPHOS promoter occupancy of Brd4 represented as reads per million per base pair (R.P.M/bp). Ref seq genes are indicated in the bottom panel. Data obtained and adapted from (Anders et al, 2014). **C**, ChIP- I-BET decreases Brd4 occupancy at the CCND2 promoter. **D**, qRT-PCR time course illustrating the dynamics of I-BET upregulated OXPHOS and down regulation of the control CCND2 genes. **E,** shBrd4 ND1-mutated cybrids display increases in OXPHOS genes. Data is representative of mean ± s.e.m., n=3. Asterisks denote $*p<0.05$ or $**p<0.01$ via Student's t-test. All I-BET treatments are delivered at a final concentration of 0.9 μM.

Figure S5. Related to Figure 6: IBET 525762A increases energetic and complex II and CoQ-linked metabolites.
A-B I-BET 525762A treatment restores ATP levels (**A**) and normalizes NADH levels (**B**) in ND1-mutated cybrids. **C,** I-BET 525762A increases transcripts in the glutamine pathway in ND1-mutated cybrids. **D-E** IBET 525762A fail to rescue ND1-mutated cybrids under galactose conditions when treated with 40 μM chloramphenicol (inhibits mitochondrial protein translation) **(D)** or 50 ng/mL ethidium bromide (depletes mitochondrial DNA) **(E)** for 9 days. Immunoblots illustrate the complete depletion of the mitochondrial-encoded CIV subunit mtCOI, whereas nuclear encoded proteins where not affected (COX5a and SDHA). All I-BET treatments are delivered at a final concentration of 0.9 μM. Data is representative of mean ± s.e.m., n=3. Asterisks denote $*p<0.05$ or $**p<0.01$ via Student's t-test.

Table S1. Related to Figure 2: Mitochondrial Proteomics. Proteomics analysis on isolated mitochondria from DMSO or I-BET (compound) treated ND1-mutant cybrids in duplicate. Fold enrichment over DMSO treated samples is indicated to the right.

Supplemental Experimental Procedures

Proteomics protein extraction and protease digestion

Mitochondrial extracts were combined 1:1 with SDS lysis buffer (4.0 % SDS w/v, 250 mM NaCl, PhosStop (Roche) phosphatase inhibitors, EDTA free protease inhibitor cocktail (Promega) and 50 mM HEPES, pH 8.5). Extracts were reduced with 5 mM DTT (57 °C for 30 minutes) and cysteine residues alkylated with iodoacetamide (14 mM) in the dark (45 min). Extracts were purified by methanol-chloroform precipitation and subsequent ice cold acetone washes. Pellets were resuspended in 8 M urea containing 50 mM HEPES, pH 8.5, and protein concentrations were measured by BCA assay (Thermo Scientific) prior to protease digestion. 200 µg of protein extracts were diluted to 4 M urea and digested with LysC (Wako) in a 1/200 enzyme/protein ratio overnight. Digests were diluted further to a 1.5 M urea concentration and trypsin (Promega,) was added to a final 1/250 enzyme/protein ratio for 6 hours at 37 °C. Digests were acidified with 20 µL of 20% formic acid (FA) to a pH ~2 and subjected to C18 solid-phase extraction (SPE) (50 mg, Sep-Pak, Waters).

Isobaric tag labeling

Isobaric labeling of peptides was performed using a 6-plex tandem mass tag (TMT) reagents (Thermo Fisher Scientific). TMT reagents (5 mg) were dissolved in 250 µl dry acetonitrile (ACN) and 10 µl was added to 100 µg of peptides dissolved in 100 µl of 200 mM HEPES, pH 8.5. After 1 hour (RT), the reaction was quenched by adding 8 µl of 5% hydroxylamine. Labeled peptides were combined, acidified with 20 µL of 20 % FA (pH ~2) and concentrated via C_{18} SPE on Sep-Pak cartridges (50 mg).

Basic pH reversed-phase HPLC (bpHrp)

TMT labeled peptides were solubilized in buffer A (5% ACN, 10 mM ammonium bicarbonate, pH 8.0) and separated by an Agilent 300 Extend C18 column (5 µm particles, 4.6 mm ID and 220 mm in length). Using an Agilent 1100 binary pump coupled with a degasser and a photodiode array (PDA) detector (Thermo Scientific), a 45 minute linear gradient from 18% to 40% acetonitrile in 10 mM ammonium bicarbonate pH 8 (flow rate of 0.8 mL/min) separated the peptide mixtures into a total of 96 fractions (37 seconds). 96 Fractions were consolidated into 12 samples in a checkerboard manner, acidified with 10 µL of 20% formic acid and vacuum dried. Each sample was re-dissolved in 5% FA, desalted via StageTips, dried via vacuum centrifugation, and reconstituted for LC-MS/MS analysis.

Oribtrap Fusion parameters

All MS analysis was performed on an Oribtrap Fusion Lumos (Thermo Fischer Scientific) coupled to a Proxeon nLC-1200 ultra-high pressure liquid chromatography (UPLC) pump (Thermo Fisher Scientific). Peptides were separated onto a packed 100 µM inner diameter column containing 0.5 cm of Magic C4 resin (5 µm, 100 Å, Michrom Bioresources) followed by 40 cm of Sepax Technologies GP-C_{18} resin (1.8 µm, 120 Å) and a gradient consisting of 6–30% (ACN, 0.125% FA) over 125 min at ~450 nl/min. The instrument was operated in data-dependent mode with a 60 s (+/- 7 ppm window) expiration time, with $FTMS^1$ spectra collected at 120,000 resolution with an AGC target of 500,000 and a max injection time of 100 ms. The ten most intense ions were selected for MS/MS and precursors were filtered according to charge state (required > 1 z). Monoisotopic precursor selection was enabled. Isolation width was set at 0.7 *m/z*. $ITMS^2$ spectra were collected at an AGC of 18,000, max injection time of 120 ms and CID collision energy of 35%. For the $FTMS^3$ acquisition, the Orbitrap was operated at 30,000 resolution with an AGC target of 50,000 and a max injection time of 250 ms and an HCD collision energy of 55%. Synchronous-precursor-selection (SPS) was enabled to include 10 MS^2 fragment ions in the $FTMS^3$ spectrum.

Data processing and MS^2 spectra assignment

A compendium of in-house software was used to convert .raw files to mzXML format, as well as to correct monoisotopic *m/z* measurements and erroneous charge state assignments. Assignment of MS/MS spectra was performed using the Sequest algorithm. A protein sequence database containing Human Uniprot database (downloaded 11/2015) as well as known contaminants such as human keratins and reverse protein sequences were appended. Sequest searches were performed using a 20 ppm precursor ion tolerance, requiring trypsin protease specificity, while allowing up to two missed cleavages. TMT tags on peptide N termini/lysine residues (+229.162932 Da) and carbamidomethylation of cysteine residues (+57.02146 Da) were set as static modifications while methionine oxidation (+15.99492 Da) was set as variable modifications. An MS^2 spectra assignment false discovery rate (FDR) of less than 1% was achieved by applying the target-decoy database search strategy and filtered using an in-house linear discrimination analysis algorithm with the following peptide ion and MS^2 spectra

metrics: XCorr, peptide ion mass accuracy, charge state, peptide length and missed-cleavages. Peptides were further filtered a 1% protein-level false discovery rate for the final dataset.

Calculation of TMT reporter ion intensities

For quantification, a 0.03 *m/z* (6-plex TMT) window centered on the theoretical *m/z* value of each reporter ion, with the maximum signal intensity from the theoretical *m/z* value was recorded. Reporter ion intensities were adjusted based on the overlap of isotopic envelopes of all reporter ions as per manufacturer specifications. Total signal to noise values for all peptides were summed for each TMT channel (300 minimum) and all values were normalized to account for variance in sample handling.

Oxygen Consumption

1.0×10^5 of the indicated cell type were seeded in an XFE-24 Seahorse plate (Seahorse Biosciences, 102340) in DMEM high glucose, 10% FBS and 1% P/S and allowed to adhere for 24 hours at 37°C and 5% CO_2. Following incubation, cells were treated with either DMSO or 0.9 µM I-BET 525762A for 24h. Medium was then removed and cells were washed with pre-warmed unbuffered DMEM without bicarbonate (Sigma, D5030) supplemented with 15 mM glucose, 2 mM sodium pyruvate, 1 mM glutamine. After the wash, 600 uL of the same buffer was added and cultured at 37°C in a non-CO_2 incubator for 1h. The Seahorse 24 optical fluorescent analyzer cartridge was prepared in the interim by adding 5 µM oligomycin, 0.5 µM FCCP, and 2 µM rotenone to each cartridge port. Oxygen consumption rates (pmole/min) and extracellular acidification rate (mpH/min) were then measured for each treatment condition at 37°C using the Seahorse Bioanalyzer instrument. After measurement, media was removed and 20 µL of RIPA buffer was added and protein concentration using BCA (Pierce 23228) was measured to normalize values.

Metabolomics

5.0×10^6 ND1-mutant cybrids were seeded in a 10 cm plate (Corning). Cells were treated with 0.9 µM I-BET 525762A or vehicle in DMEM with 10 mM galactose, 10% FBS, 1% P/S and 1mM glutamine and incubated for 16 hrs at 37°C with 5% CO_2. Following incubation, cells were harvested on dry ice with 1 mL chilled 80% HPLC-grade methanol (Fluka Analytical). Cell mixture was incubated for 15 min on dry ice prior to centrifugation at 18,000 x g for 10 min at 4°C. Supernatant was retained and remaining cell pellet was resuspended in 800 µL chilled 80% methanol and centrifuged. Supernatant was combined with the previous retention and was lyophilized using a SpeedVac (Thermo Fisher). Lyophilized samples were resuspended in 20 µL ultrapure water and subjected to metabolomics profiling using the AB/SCIEX 5500 QTRAP triple quadrupole instrument. Data analysis was performed using the GiTools software.

Complex Activities

For complex II measurements, succinate dehydrogenase activity (CII activity) was measured using 100 µg of isolated mitochondrial resuspended in 950 µL CI/CII buffer (25 mM K_2HPO_4 pH 7.2, 5 mM $MgCl_2$, 3 mM KCN, 2.5 mg/ml BSA, 10 mM succinate, 0.03 mM DCPIP (Sigma, 33125), 2 µg/ml antimycin A and 5 µM rotenone). Isolated mitochondria were incubated for 10 min at 37°C. Reaction was initiated by adding 15 µL of 10 mM ubiquinone and the decrease in absorbance at 600 nm (ε= 19.2 mM-1cm-1) was measured for 4 minutes at 37ºC in a 96 well plate (Corning, 3603) spectrophotometrically (Fluostar Omega). Specificity of complex II activity was verify by running the assay after the addition of 50mM TTFA (2 thenoyltrifluoroacetone) before starting the reaction. Cytochrome c reductase activity (CIII activity) was measured at 550 nm (ε= 21 mM-1cm-1) in a mix containing 25 mM K2HPO4 pH 7.2, 5 mM MgCl2, 3 mM KCN, 2.5 mg/ml BSA, 0.3 mg/ml N-dodecyl-β -D-maltoside (DDM), 5 µM rotenone and 0.06 mM freshly prepared ubiquinol-2 (UQ2H2). Specificity of complex III activity was verified by inhibiting the complex with 0.1mM Antimycin A. CIV activity was measured spectrophotometrically. Briefly, 2.0×10^6 cells were trypsinized and resuspended in 10mM K_2HPO_4 buffer pH 7.4 containing 0.025% n-Dodecyl β-D-maltoside (DDM) (Sigma, D5172). After 2 minutes of permeabilization, 1 mg/ml of reduced cytochrome C (Sigma, C2506) was added and the decrease in absorbance at 550 nm was measured for 3 minutes at 37ºC in a 96 well plate using the spectrophotometer. Specificity of complex IV activity was verified by adding 10mM KCN. Complex V activity was measured at 340 nm (ε= 6.22 mM-1cm-1) in a mix containing H-Mg buffer, 0.3mM NADH, 2.5mM phosphoenolpyruvic acid, 50ug/ml pyruvate kinase, 50ug/ml lactate dehydrogenase, 2ug/ml Antimycin A and 2.5mM ATP. Specificity of complex V activity was verified by adding 2ug/ml Oligomycin. Citrate synthase activity was measured at 412 nm (ε= 13.8 mM-1cm-1) in a mix containing Tris-HCL buffer 75mM, DTNB (5,5′-dithiobis 2-nitrobenzoic acid) 0.1mM, triton X-100 0.1%, Acetyl CoA 0.4mM. Reaction was started by adding 0.5mM oxaloacetate.

Chromatin Immunoprecipitation
A minimum of $1.0x10^7$ cells were treated with either vehicle or 0.9 µM I-BET 525762A for 24 h at 37°C with 5% CO_2. Cells were then crosslinked with 1% formaldehyde (Sigma) for 10 min followed by quenching with 0.125 M glycine for 5 min. Cells were lysed with ChIP lysis buffer (50 mM HEPES, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, and Complete protease inhibitors (Roche) and sonicated in a Diagenode Bioruptor for 5 cycles for 5 min with a duty cycle of 30 s "on" and 30 s "off." Samples were immunoprecipitated with specific antibodies to Brd4 (Bethyl, A301-985A50), PGC-1α (Santa Cruz, sc13067) and normal rabbit IgG (Cell Signaling, 2729). Brd4 enriched chromatin was analyze by qPCR (Biorad) with the following primers: ATP1A3 promoter: (F) 5'-ACGCGGGCATATGAGGAG-3, (R) 5'-AGCACCTACCCCCATCTT-3', SDHD promoter: (F) 5'-TCACCCAGCATTTCCTCTTC-3', (R) 5'-CTGGAGGCTACGCTAAGCAC-3', CYCS promoter: (F) 5'- CTAAGTTGAAGCTTTCGTT-3', (R) 5'-AGACCATGGAGATTTGGC-3', COX5a promoter: (F) 5'-CAACCCGCGAGCTTACA-3', (R) 5'-GTATATTGTGTCCTCGGAC-3', CCND2 promoter: (F) 5'-CCCGCCCCAAAGCTTATT-3', (R) 5'-CTCTTCCTGTCTGATTCAGCTTAGG -3'. Computational Brd4 genome-wide ChIP analyses were performed using the data obtained from Anders et al., 2014. Briefly files were analyzed using the Integrative Genomics Viewer (IGV 2.3.67) software. A custom list of OXPHOS promoters obtained from Panther Gene Ontology (GO: 0006119) was used to filter Brd4 binding peaks and average peak values ± 1000bp of the TSS was obtained using the GITools 2.2.3. software.

Immunoblot
Cells were harvested in RIPA buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.1% Sodium Deoxycholate, 0.1% SDS, 140 mM NaCl, 1X protease inhibitor cocktail, 1mM PMSF) and proteins were quantified using the BCA assay (Pierce 23228). The following antibodies were used for western blot analysis: anti-Cox5a (Abcam, ab110262), anti-Sdha (Abcam, ab1715), anti-mtCO1 (Abcam, ab14705), anti-CYCS (Abcam, 167568), anti-β-Tubulin (Cell Signaling, 2146), anti-Ndufa9 (Abcam, ab14713), anti-Uqcrc2 (Abcam, ab14745), anti-Brd4 (Bethyl, A301-985A50), anti-Flag (Sigma, F1804) and anti-β-actin (Cell Signaling, 4967). Densitometry was performed using ImageJ v1.49 software.

Gene Expression
Total RNA was isolated with Trizol (Invitrogen, 15596-026). Two micrograms of RNA was used to generate complementary DNA (cDNA) with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368813) following the manufacturer's protocol. For gene expression analysis, cDNA samples were mixed with Sybr Green qPCR mastermix (Applied Biosystems, 4309155) and were analyzed by a CFX 384 Real-Time system (Bio-Rad). All primers and sequences are available upon request.

Blue native gel electrophoresis (BNGE)
Digitonin (DIG) at 4 g per g mitochondrial protein was used to solubilize the ETC complexes and 100 µg were applied and run on pre-cast 4%–16% first-dimension gradient BN gels (NativePAGE™ NOVEX life technologies) as described elsewhere (Lapuente-Brun et al., 2013). After electrophoresis, the complexes were electroblotted onto PVDF membranes and sequentially probed with specific antibodies, CIII (anti-core2) ab203832, CIV (anti-COI) ab203912, CII (SDHA 70kda subunit) ab137040, CV (anti-ATP5a) ab14748, and VDAC1 cell signalling D73D12.

Glutamine Oxidation
$5.0x10^4$ ND1 cells were seeded in a 24-well plate (Corning) and treated with either DMSO or 0.9 µM I-BET 525762A for 24 h. Next day, media containing 0.5 µCi of ^{14}C Glutamine (Perkin Elmer NEC451050UC) was added and cells were incubated at 37ºC for 3 h and the reaction was stopped by adding 200 uL of 1 M Perchloric acid. Next, 2-phenylethylamine saturated Whatman paper was placed under the tube cap in order to trap radiolabeled CO_2 during overnight incubation. Finally, the whatman paper was placed in scintillation liquid and radioactive counts were measured in a scintillation counter (Perkin Elmer, 2900TR).

Cell Imaging
Cells were imaged using the Leica inverted microscope (Leica, DMI 6000B) and images were acquired at with the Leica Application Suite V4.0 software.

ATP levels

ATP levels were measure in 96 well plates (Corning, 3603) using the ADP/ATP Ratio Bioluminescence Assay Kit according to manufacture instructions (Biovision K255-200).

NADH levels

NADH levels were measured according to the manufacturer's protocol (EnzyChrom E2ND-100). Briefly, $1.0x10^5$ cells that were either treated with 0.9 μM I-BET 525762A or vehicle, were homogenized in NADH extraction buffer. Optical density was measured at 565 nm spectrophotometrically using the Fluostar Omega Spectrophotometer and software.

Statistical Analysis

All statistics are described in figure legends. In general, for two experimental comparisons, a two-tailed unpaired Student's *t*-test was used. For multiple comparisons, one-way ANOVAs with Tukey, Newman–Keuls or Dunnett post-test were applied. Three replicates per treatment were chosen as an initial sample size unless otherwise stated in figure legends. All western blot analyses were repeated at least three times. Statistical significance is represented by asterisks corresponding to $*P < 0.05$ or $**P < 0.01$