Supplemental Information

TCA cycle and mitochondrial membrane potential are necessary for diverse biological functions

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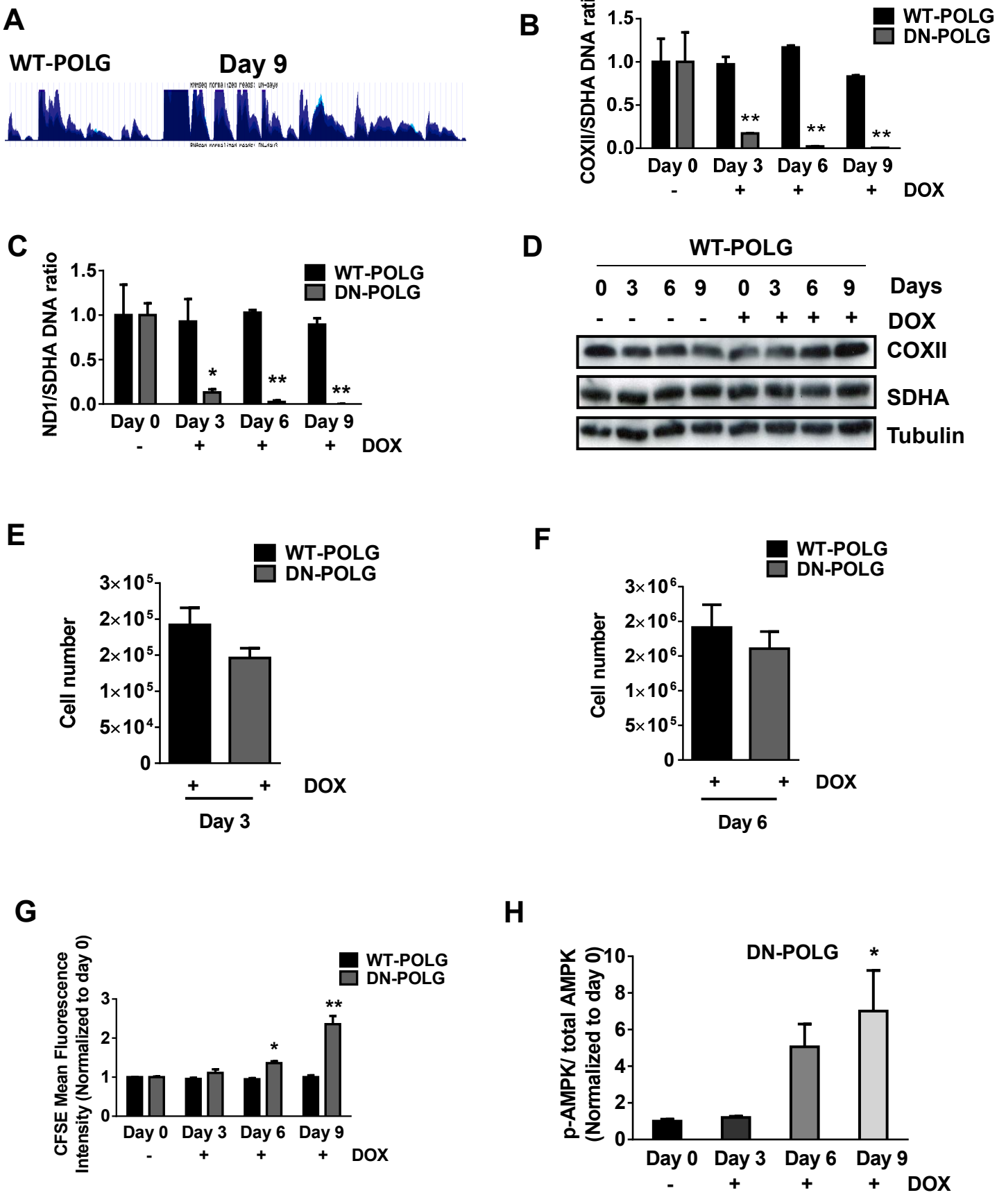
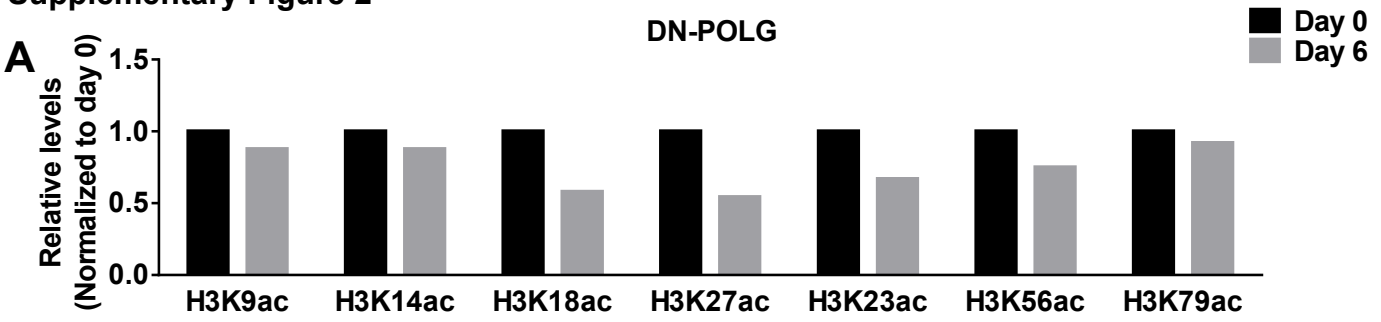
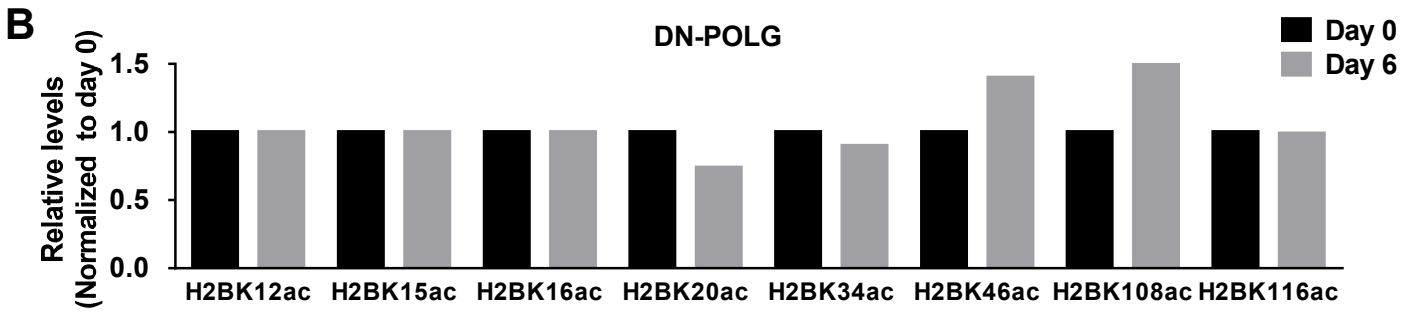
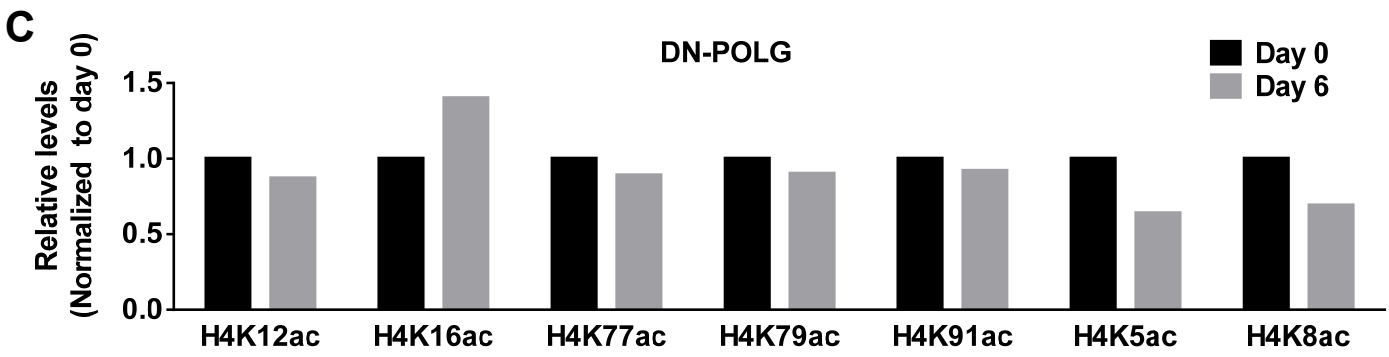
Supplementary Figure 1

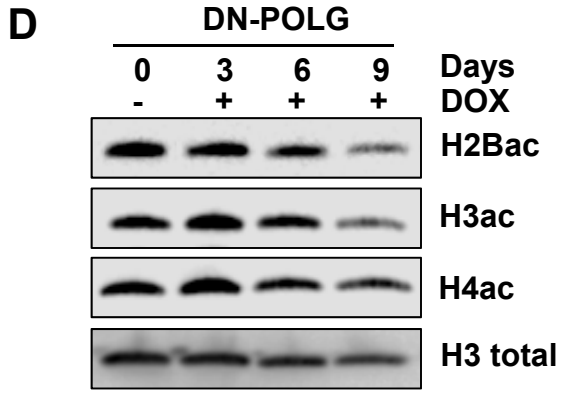


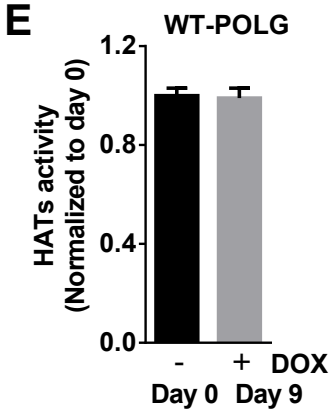
Supplementary Figure 2

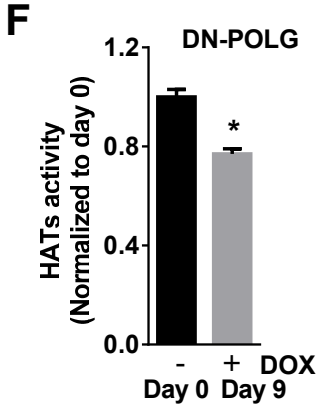


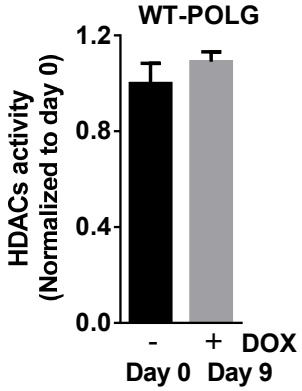


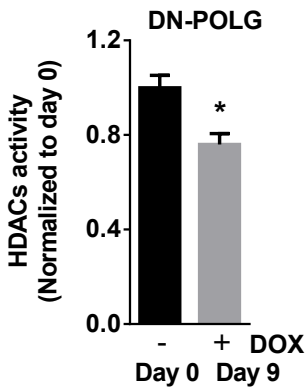




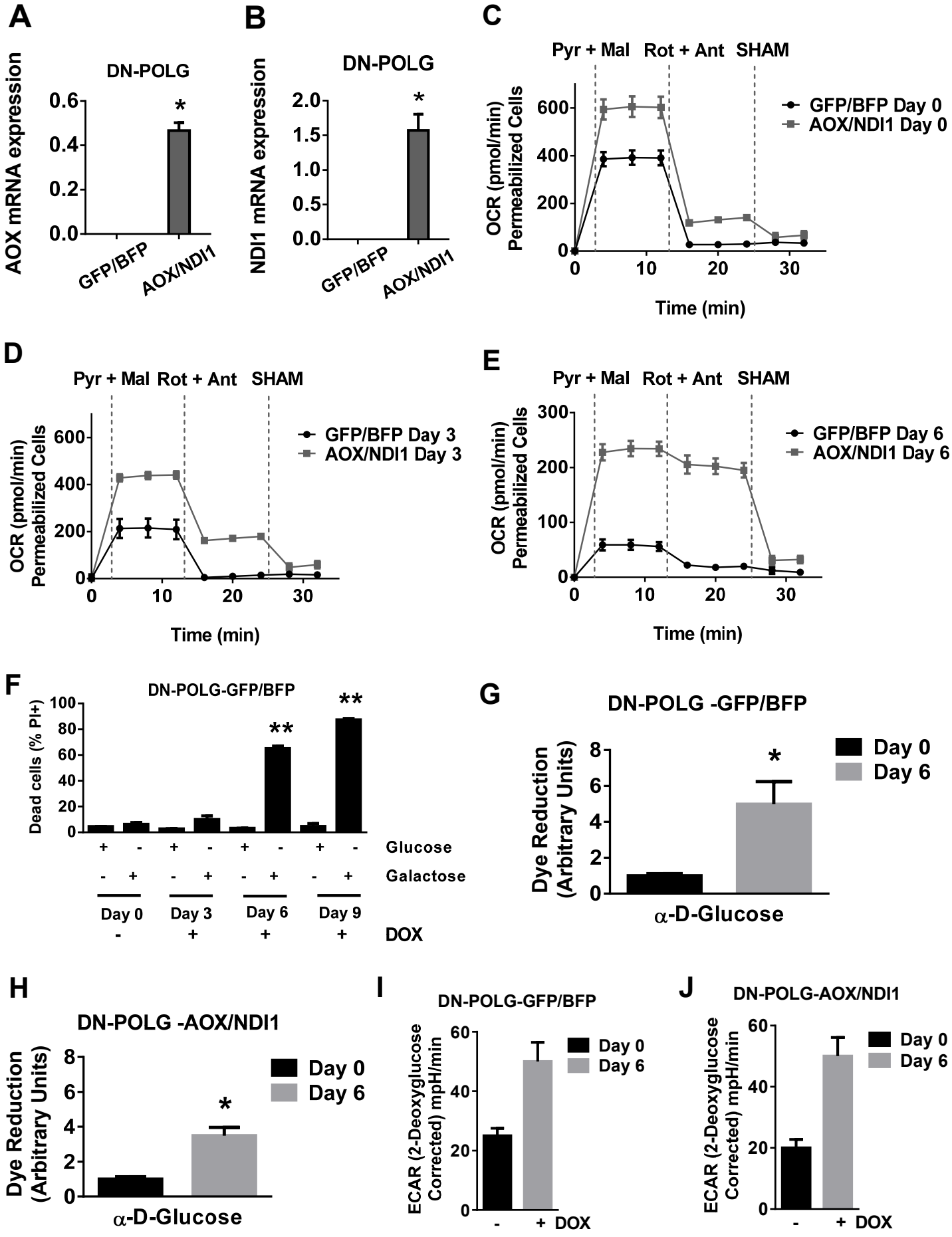




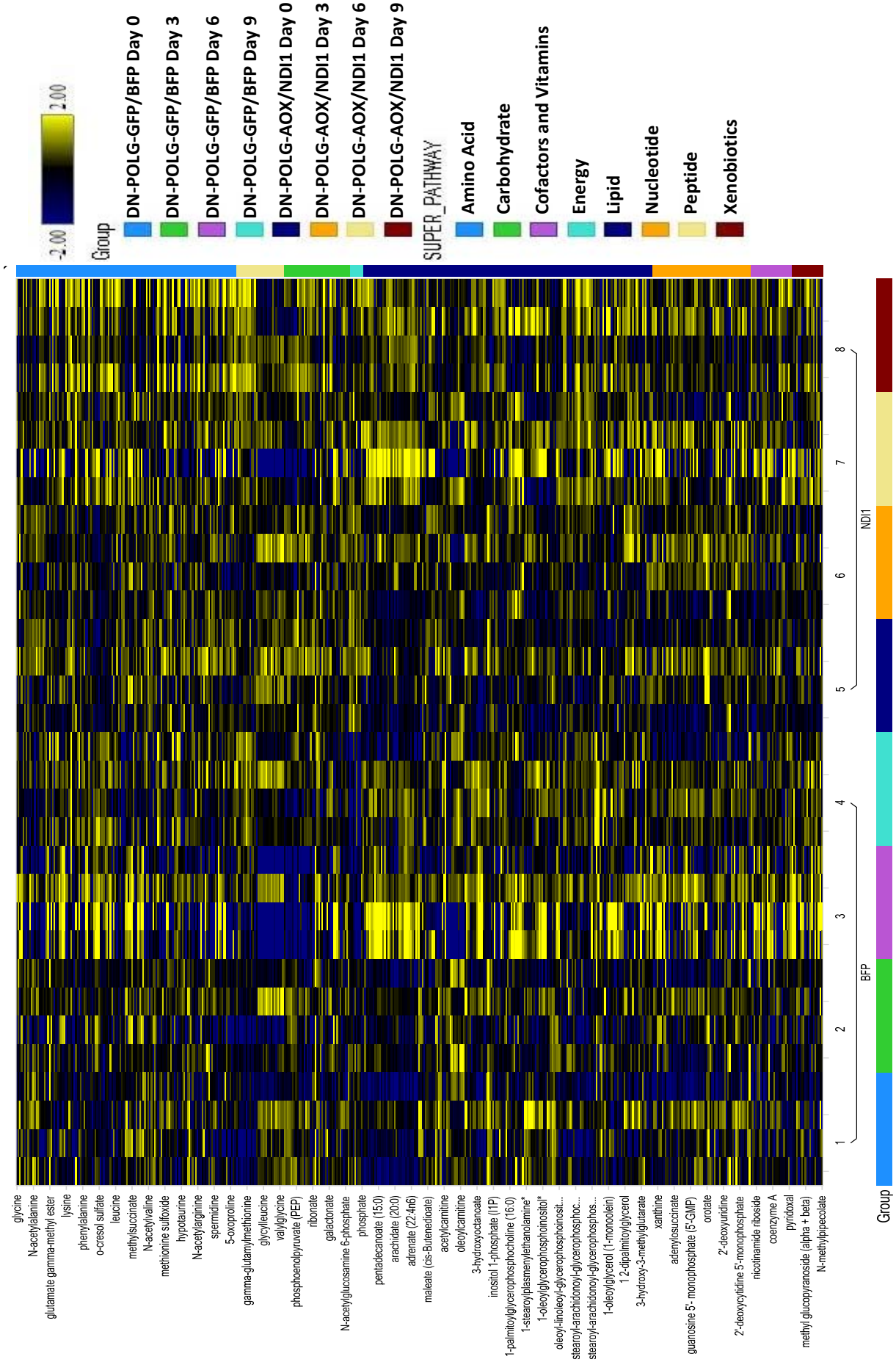




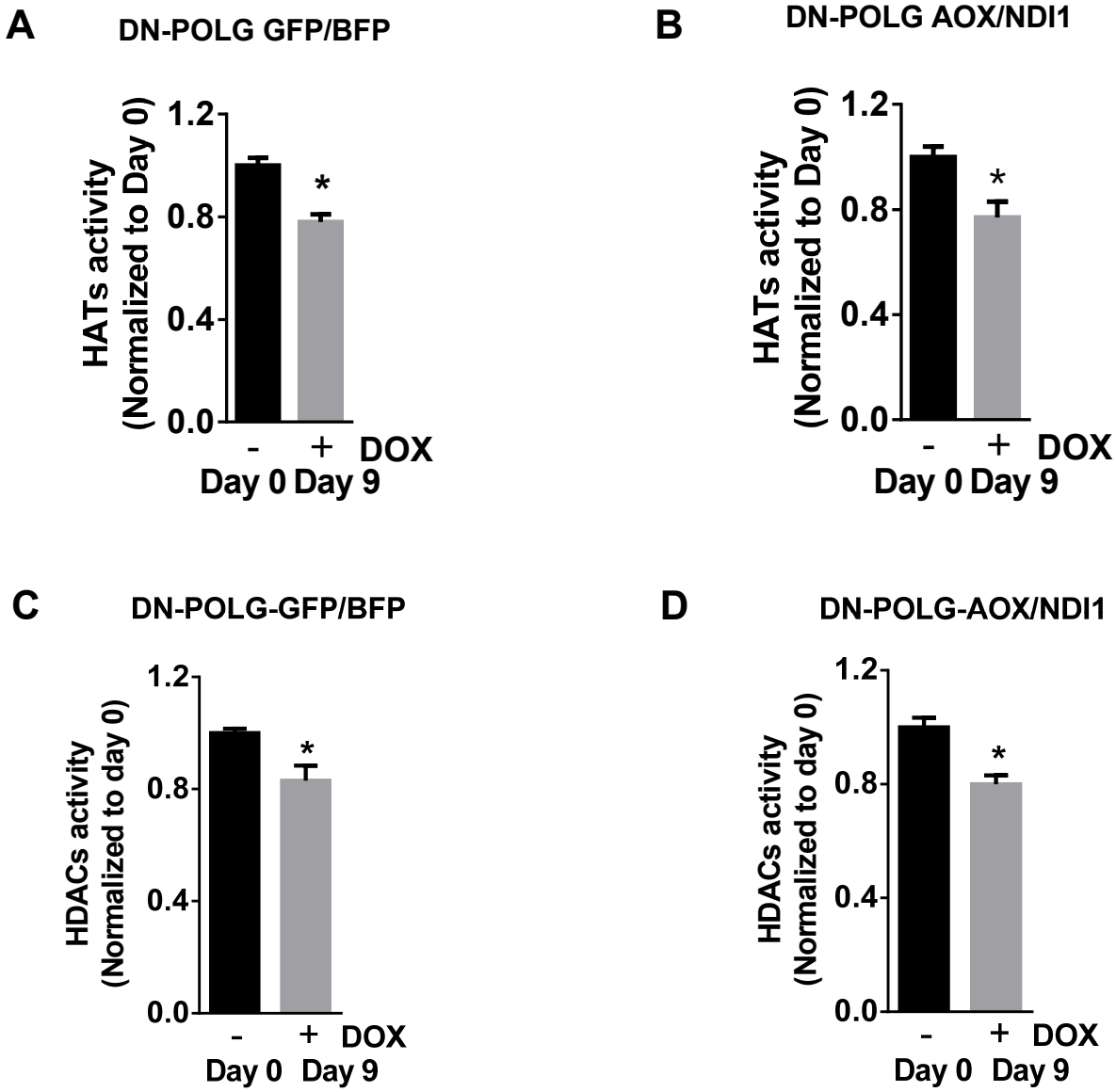
Supplementary Figure 3



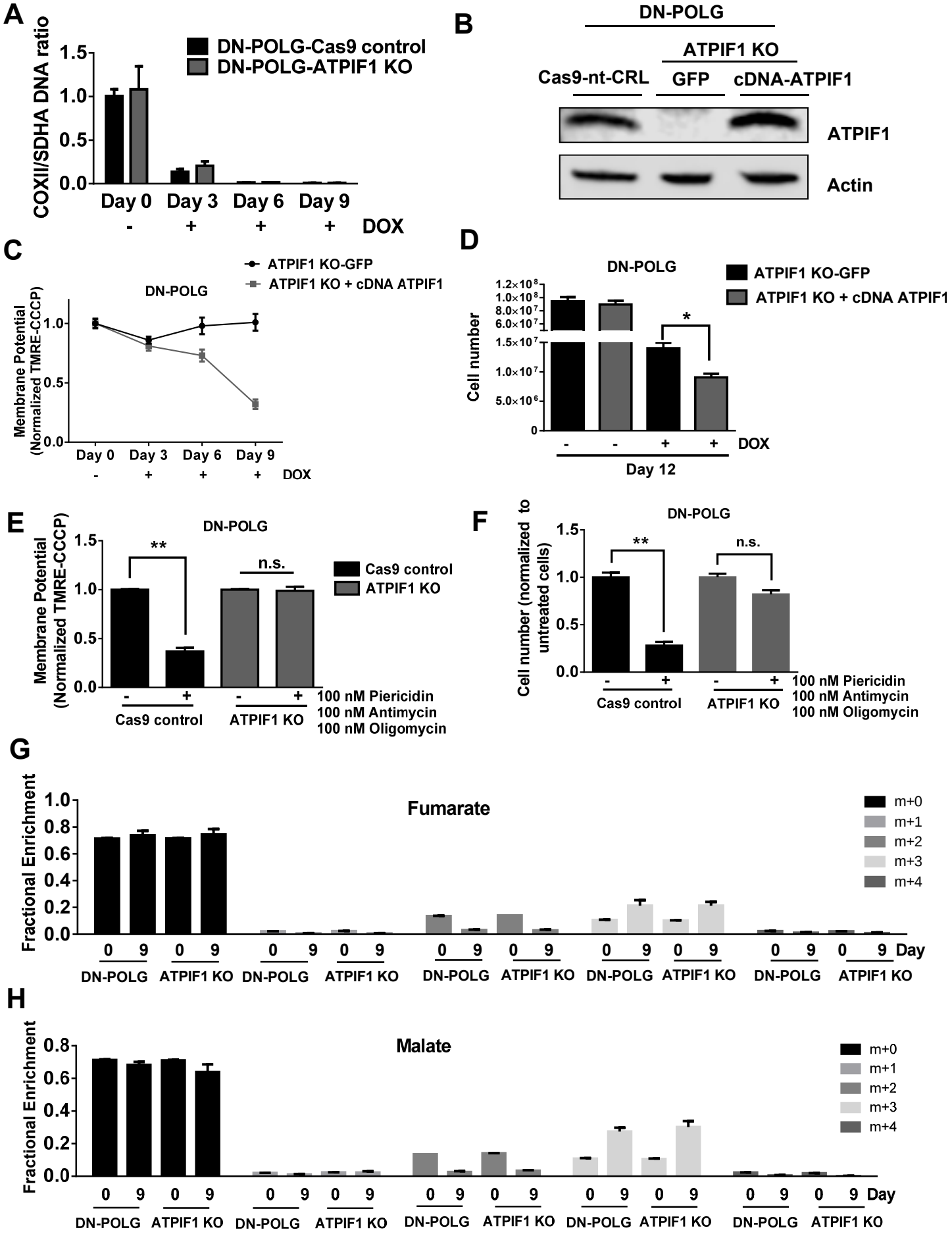
Supplementary Figure 4



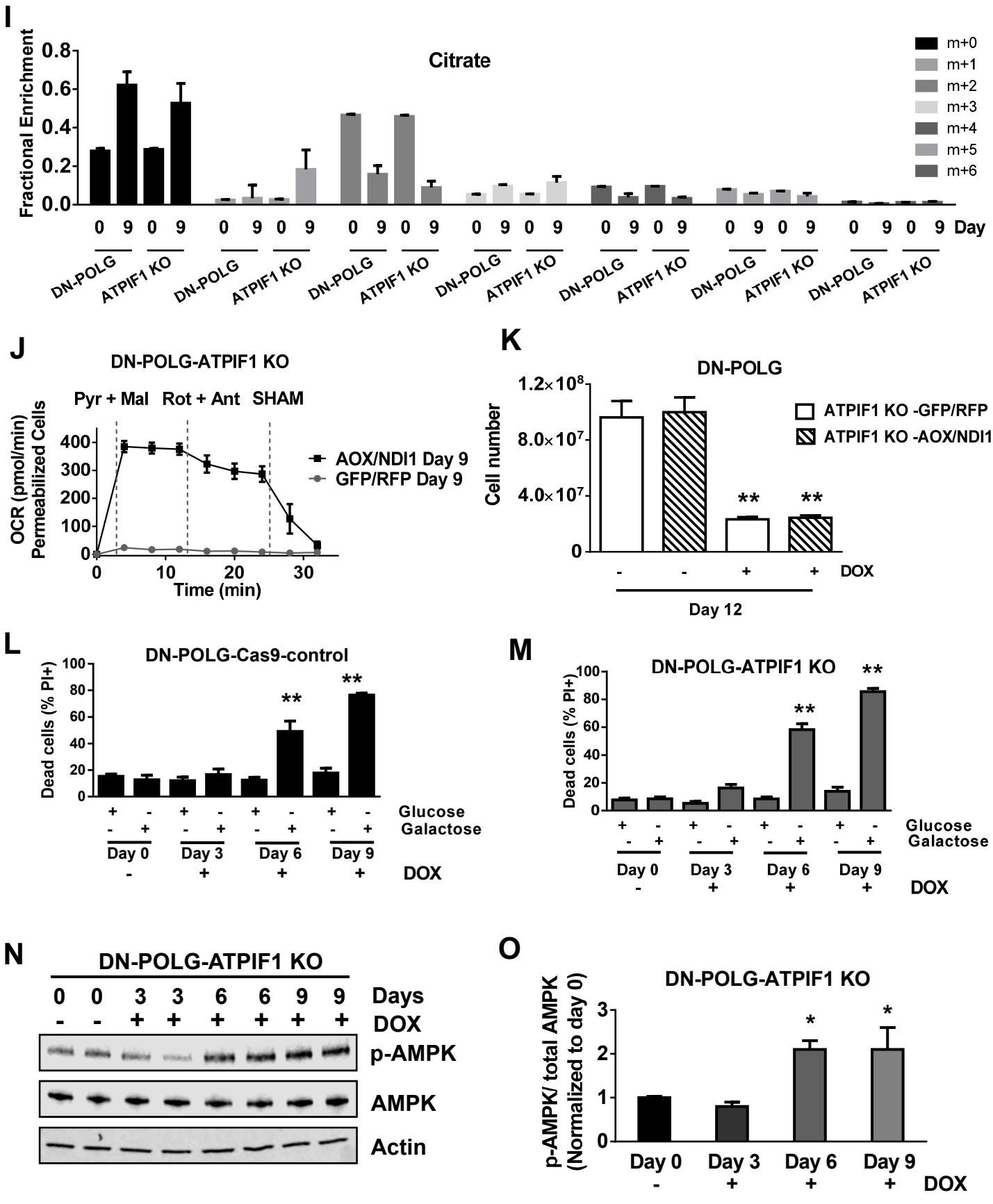
Supplementary Figure 5



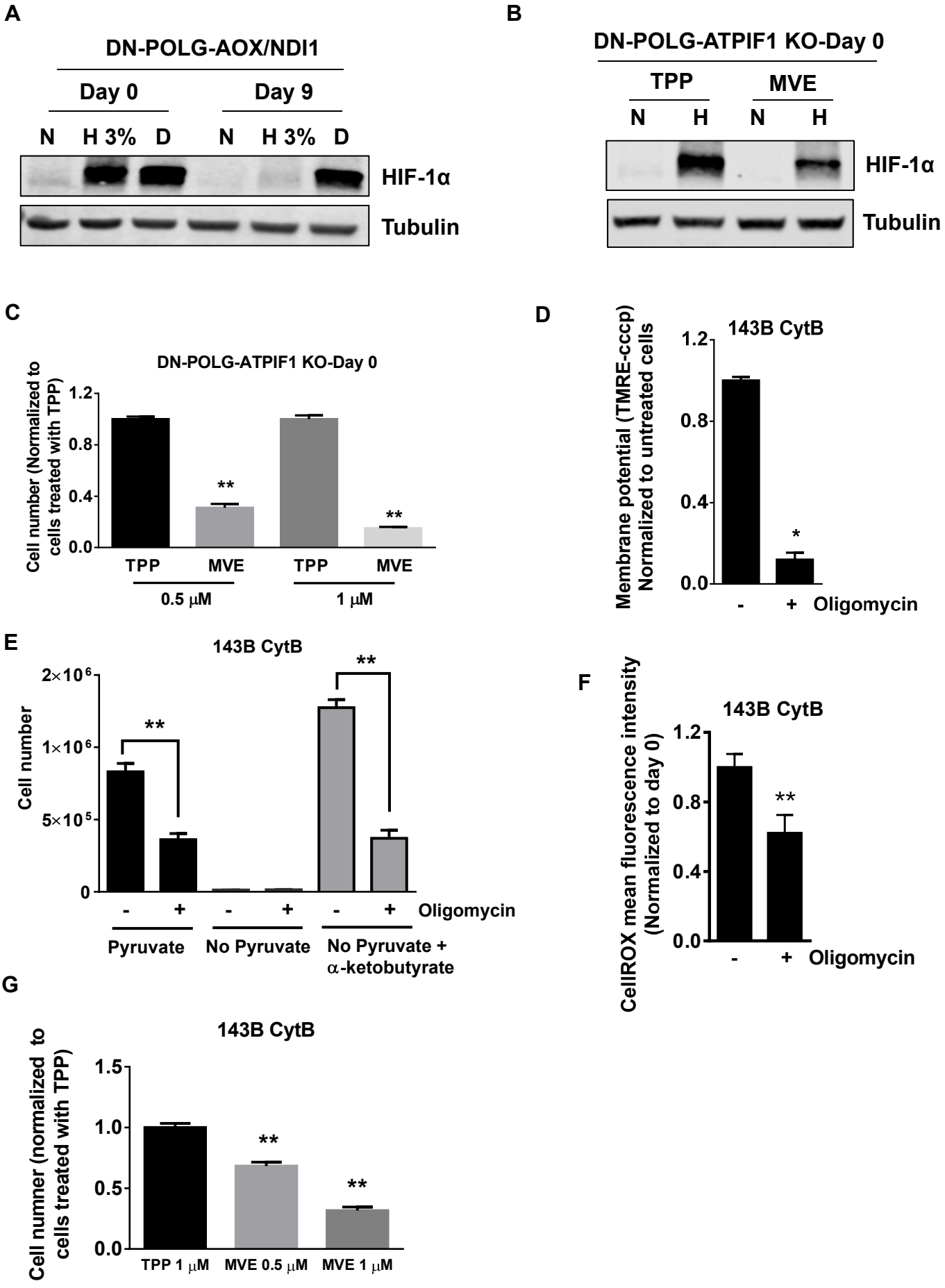
Supplementary Figure 6



Supplementary Figure 6



Supplementary Figure 7



Supplemental Figure Legends

Figure S1. Doxycycline induced expression of dominant negative POLG depletes mtDNA resulting in diminished cell proliferation and AMPK activation, related to Figure 1.

(A) RNA-sequence analysis of mitochondrial transcripts in three independent samples of WT-POLG cells treated with doxycycline (10 ng/ml) for 9 days.

(B-C) mtDNA content was monitored in WT and DN-POLG cells untreated or treated with doxycycline (10 ng/ml) for 3, 6 and 9 days by measuring the relative copy numbers of the mitochondrial encoded genes COXII (B) and ND1 (C) using quantitative PCR. Mitochondrial gene content is expressed relative to SDHA gene content and relative to untreated cells. Error bars represent data ± SEM (n=3).

(D) Western blot analysis of the levels of COXII, SDHA and tubulin in WT-POLG cells untreated or treated with doxycycline (10 ng/ml) for 3, 6 and 9 days.

(E-F) WT and DN-POLG cell numbers were counted by flow cytometry using fluorescent beads at days 3 (E) and 6 (F) of doxycycline (10 ng/ml) treatment. Error bars represent data ± SEM (n=3).

(G) WT and DN-POLG untreated or treated with doxycycline (10 ng/ml) for 3, 6 and 9 days were labeled with CFSE (5 μM) for 3 days. The dilution of the CFSE was analyzed by flow cytometry. Error bars represent data ± SEM (n=4).

(H) Western blot quantification of p-AMPK levelsrelative to the total AMPK expression in DN-POLG cells untreated or treated with doxycycline (10 ng/ml) for 3, 6 and 9 days. Error bars represent data ± SEM (n=3).

* indicates significance $p < .05$. ** indicates significance $p < 0.01$ throughout figure S1.

Figure S2. Inducible depletion of mtDNA results in specific histone modifications, related to Figure 2.

(A-C) Cells were differentially labeled by growing them in medium containing light (Day 6) or heavy (Day 0) amino acids (SILAC). Histones were extracted and modifications in histone 3 (A), histone 2B (B) and histone 4 (C) were analyzed by quantitative proteomics (LC-MS/MS).

(D) Acetylation state of H2B, H3 and H4 in DN-POLG cells untreated or treated with doxycycline (10 ng/ml) for 3, 6 and 9 days. Total H3 levels were used as loading control.
(E-F) Histones acetyltransferases (HATs) activity was measured in WT-POLG (E) and DN-POLG (F) cells untreated or treated with doxycycline (10 ng/ml) for 9 days. Error bars represent data ± SEM (n=3).
(G-H) Histone deacetylases (HDACs) activity was measured in WT-POLG (G) and DN-POLG (H) cells untreated or treated with doxycycline (10 ng/ml) for 9 days. Error bars represent data ± SEM (n=3).
* indicates significance $p < .05$ throughout figure S2.

Figure S3. NDI1 and AOX expression restores oxygen consumption but not dependency on glycolysis for survival in inducible mtDNA depleted cells, related to figure 3.

(A-B) Cellular RNA was extracted and the relative mRNA abundance of AOX (A) and NDI1 (B) was determined by RT-qPCR in DN-POLG cells expressing AOX and NDI1 and control cells. Error bars represent data ± SEM (n=3).
(C-E) Complex I (2 mM malate, 10 mM pyruvate, 10 mM ADP) driven oxygen consumption rate of saponin permeabilized DN-POLG-GFP/BFP and DN-POLG-AOX/NDI1 cells treated with doxycycline (10 ng/ml) for 0 (C), 3 (D) and 6 (E) days. Oxygen consumption was measured in mitochondrial assay buffer. Rotenone (1 μM) and antimycin (1 μM) were used to inhibit complex I and III respectively. SHAM (2 mM) was used to inhibit AOX activity. Error bars represent data ± SEM (n=3).
(F) DN-POLG-GFP/BFP cells untreated or treated with doxycycline (10 ng/ml) for 3, 6 and 9 days were grown in media containing 10 mM glucose or 10 mM galactose for 48h following PI staining and analysis of cell death by flow cytometry. Error bars represent data ± SEM (n=3).
(G-H) Glucose utilization by DN-POLG-GFP/BFP (G) and DN-POLG-AOX/NDI1 (H) cells untreated or treated with doxycycline (10 ng/ml) for 6 days. Error bars represent data ± SEM (n=3).
(I-J) The extracellular acidification rate (ECAR) was measured in DN-POLG-GFP/BFP (I) and DN-POLG-AOX/NDI1 (J) cells untreated or treated with doxycycline (10 ng/ml)

for 6 days. Data show a representative experiment (from 3 independent experiments). Error bars ± SD (n=5).
* indicates significance p < .05. ** indicates significance p <0.01 throughout figure S3.

Figure S4. Heat map of metabolites in inducible mtDNA depleted cells expressing NDI1 and AOX, related to figure 4

The heat map of metabolites that are significantly upregulated or downregulated when DN-POLG-GFP/BFP or DN-POLG-AOX/NDI1 were treated with doxycycline (10 ng/ml) for 3, 6 and 9 days. Levels are relative to untreated cells. A yellow-blue color scale depicts the abundance of the metabolites (Yellow: high, Blue: low). Data represent the average of 4 independent experiments.

Figure S5. NDI1 and AOX expression does not rescue HATs and HDACs activities, related to figure 5.

(A-B) Histones acetyltransferases (HATs) activity was measured in DN-POLG-GFP/BFP (A) and DN-POLG-AOX/NDI1 (B) cells untreated or treated with doxycycline (10 ng/ml) for 9 days. Error bars represent data ± SEM (n=3).
(C-D) Histone deacetylases (HDACs) activity was measured in DN-POLG-GFP/BFP (C) and DN-POLG-AOX/NDI1 (D) cells untreated or treated with doxycycline (10 ng/ml) for 9 days. Error bars represent data ± SEM (n=3).
* indicates significance p < .05 throughout figure S5.

Figure S6. Loss of ATPIF1 increases mitochondrial membrane potential in inducible mtDNA depleted cells, related to figure 6.

(A) mtDNA content was monitored by measuring the relative copy numbers of mitochondrial encoded gene COXII using quantitative PCR in DN-POLG-Cas9-control cells and DN-POLG-ATPIF1 KO cells untreated or treated with doxycycline (10 ng/ml) for 3, 6 and 9 days. Mitochondrial gene content is expressed relative to SDHA gene content and relative to untreated cells. Error bars represent data ± SEM (n=3).

(B) Western blot analysis of ATPIF1 protein in DN-POLG-Cas9-nt-control, DN-POLG-ATPIF1 KO cells infected with the empty vector (GFP) and DN-POLG-ATPIF1 KO cells overexpressing the cDNA of ATPIF1. Actin levels were used as loading control.

(C) Mitochondrial membrane potential assessed by TMRE (50nM) staining and corrected by CCCP (50 μM) of DN-POLG-ATPIF1 KO-GFP and DN-POLG-ATPIF1 KO + cDNA-ATPIF1 cells untreated or treated with doxycycline (10 ng/ml) for 3, 6 and 9 days. Error bars represent data ± SEM (n=3).

(D) DN-POLG-ATPIF1 KO-GFP and DN-POLG-ATPIF1 KO + cDNA-ATPIF1 cells were grown in the presence or absence of doxycycline (10 ng/ml) for 12 days and cell number was assessed. Error bars represent data ± SEM (n=3).

(E) Mitochondrial membrane potential assessed by TMRE (50nM) staining and corrected by CCCP (50 μM) of DN-POLG-Cas9-nt-control and DN-POLG-ATPIF1 KO cells untreated or treated with piericidine (100 nM), antimycin (100 nM) and oligomycin (100 nM) for 6h. Error bars represent data ± SEM (n=3).

(F) DN-POLG-Cas9-nt-control and DN-POLG-ATPIF1 KO cells were exposed to piericidine (100 nM), antimycin (100 nM) and oligomycin (100 nM) for 72h and cell number was assessed. Error bars represent data ± SEM (n=3).

(G-I) DN-POLG and DN-POLG-ATPIF1 KO cells untreated or treated with doxycycline (10 ng/ml) for 9 days were labeled for six hours with [U-^{13}C]glucose resulting in ^{13}C enrichment in fumarate (G), malate (H) and citrate (I). Error bars represent data ± SEM (n=3).

(J) Complex I (2 mM malate, 10 mM pyruvate, 10 mM ADP) driven oxygen consumption rate of saponin permeabilized DN-POLG-ATPIF1 KO-GFP/RFP and DN-POLG-ATPIF1 KO-AOX/NDI1 cells treated with doxycycline (10 ng/ml) for 9 days. Oxygen consumption was measured in mitochondrial assay buffer. Rotenone (1 μM) and antimycin (1 μM) were used to inhibit complex I and III respectively. SHAM (2 mM) was used to inhibit AOX activity. Error bars represent data ± SEM (n=3).

(K) DN-POLG-ATPIF1 KO-GFP/RFP cells and DN-POLG-ATPIF1 KO-AOX/NDI1 cells were grown in the presence or absence of doxycycline (10 ng/ml) for 12 days and assessed for cell number. Error bars represent data ± SEM (n=3).

(L-M) DN-POLG-Cas9-control (L) and DN-POLG-ATPIF1 KO cells (M) untreated or treated with doxycycline (10 ng/ml) for 3, 6 and 9 days were grown in media containing 10 mM glucose or 10 mM galactose for 48h following PI staining and analysis of cell death by flow cytometry. Error bars represent data ± SEM (n=3).

(N) The blots show the levels of p-AMPK, AMPK and actin in DN-POLG-ATPIF1 KO cells untreated or treated with doxycycline (10 ng/ml) for 3, 6 and 9 days.

(O) The histogram shows the quantification of the western blots performed to assess the phosphorylation state of AMPK in DN-POLG-ATPIF1 KO cells. Data is represented relative to total levels of AMPK and the mean value in untreated cells. Error bars represent data ± SEM (n=3).

*indicates significance $p<0.05$, **indicates significance $p<0.01$ throughout supplementary figure S6.

Figure S7. Mitochondrial ROS are necessary for cell proliferation and HIF-1 activation, related to figure 7.

(A) DN-POLG-AOX/NDI1 cells untreated or treated with doxycycline (10 ng/ml) for 9 days were placed in normoxia (21% O_2), hypoxia (3% O_2) or treated with 1 mM DMOG for 4 hours. Hypoxic induction of HIF-1α was analyzed by western blot. Tubulin levels were used as loading control.

(B) DN-POLG-ATPIF1 KO cells in the absence of doxycycline were pretreated with 1 μM TPP or MVE and subsequently placed in normoxia (21% O_2), hypoxia (1.5% O_2) or treated with 1 mM DMOG (21% O_2) for 4 hours. Hypoxic stabilization of HIF-1α was analyzed by western blot. Tubulin levels were used as loading control.

(C) DN-POLG-ATPIF1 KO cells in the absence of doxycycline were exposed to 0.5 μM or 1 μM TPP or MVE for 3 days and cell number was assessed. Error bars represent data ± SEM (n=3).

(D) Mitochondrial membrane potential assessed by TMRE (50nM) staining and corrected by CCCP (50 μM) of 143B CytB cells untreated or treated with oligomycin (20 nM) for 24h. Error bars represent data ± SEM (n=3).

(E) 143B CytB cells grown in the presence or absence of pyruvate (1 mM) and α-ketobutyrate (1 mM) were treated with oligomycin (20 nM) for 96h and cell number was assessed. Error bars represent data ± SEM (n=3).
(F) ROS levels were measured in 143B CytB cells untreated or treated with oligomycin (20 nM) for 48h. Data was normalized to the mean value in untreated cells. Error bars represent data ± SEM (n=3).
(G) 143B CytB cells were exposed to 1 μM TPP or 0.5 μM/ 1 μM MVE for 72h and cell number was assessed. Error bars represent data ± SEM (n=3).
*indicates significance $p<0.05$, **indicates significance $p<0.01$ throughout supplementary figure 7.

Table S1. Metabolomics of mtDNA depleted cells, related to figure 4. DNPOLG control GFP/BFP cells and DNPOLG AOX/NDI1 cells were treated with doxycycline for 9 days and metabolomic analysis was done at day 3, 6, and 9.

Supplemental Experimental Procedures

Generation of Cell Lines and Culture. WT-POLG and DN-POLG T-REx293 cells were grown in ρ^o media (Dulbecco's Modified Eagle's Medium containing 4.5 g/L glucose, 4 mM L-glutamine, 10% fetal bovine serum, 1 mM sodium pyruvate, 100 µg/ml uridine, 1% HEPES, 1% antibiotic-antimycotic (Gibco), 150 µg/ml hygromycin and 5 µg/ml blasticidin) at 37 °C with 5% CO_2. AOX, NDI1 and control pWPI vectors containing GFP and BFP (Cannino et al., 2012) as well as ATPIF1 mutated cDNA, NDI1 and pCDH vectors, containing GFP and RFP, were transfected into 293T cells using lipofectamine 2000 (Invitrogen) along with pMD2.G and psPAX2 packaging vectors to produce Control-GFP, Control-BFP, Control-RFP, AOX-GFP, NDI1-BFP, ATPIF1-GFP or NDI1-RFP lentivirus. Parental DN-POLG cells were infected with either Control-GFP and BFP lentivirus or AOX-GFP and NDI1-BFP lentivirus to generate control-DN-POLG-GFP/BFP and DN-POLG-AOX/NDI1 cells. Selection of the double positives GFP/BFP expressing Control-DN-POLG and DN-POLG-AOX/NDI1 was done by periodic fluorescence-activated cell sorting (FACS) for GFP/BFP-positive cells using the BD FASCAria. Cells were routinely checked for GFP and BFP expression using FACS to ensure high AOX and NDI1 expression. DN-POLG-ATPIF1 KO cells were generated using the LentiCRISPR plasmid, which expresses Cas9 along with gene-specific RNA guides (gRNAs). The primers used to clone the gRNA were:
ATPIF1-Guide-F, CACCGAATGGCAGTGACGGCGTTGG; ATPIF1-Guide-R AAACCCAACGCCGTCACTGCCATTC. Cells expressing only Cas9 and cells expressing Cas9 and a non-targeting gRNA were used as controls. The primers used to clone the non-targeting gRNA were: nt-F, CACCGGTAGCGAACGTGTCCGGCGT; nt-R, AAACACGCCGGACACGTTCGCTACC (Wang et al., 2014). To reconstitute the cDNA of ATPIF1, site directed mutagenesis was performed in the PAM sequence to avoid Cas9 recognition using the Quick Change Kit from Agilent. DN-POLG-ATPIF1 KO cells were infected with either control-GFP and RFP lentivirus or AOX-GFP and NDI1-RFP lentivirus to generate DN-POLG-ATPIF1 KO GFP/RFP and DN-POLG-ATPIF1 KO AOX/NDI1 cells.

Proliferation and Cell Viability analysis $2.0x10^4$ cells were plated on 24mm dishes. Cells were expanded and counted 3, 6, 9 and 12 days after plating in the presence or absence of doxycycline using AccuCount Fluorescent Particles from Fisher. Cell viability percentage was determined by DAPI or PI staining and analyzed by flow cytometry. To monitor distinct generations of proliferating cells, carboxyfluorescein succinimidyl ester (CFSE) was used to stain cells for 3 days and dilution of the dye was detected by flow cytometry. All flow cytometric analyses were performed using a LSR II flow-cytometer (Becton Dickinson, Franklin Lakes, NJ USA).

Mitochondrial Membrane Potential Measurements. The membrane potential of mitochondria was analyzed with the potential-dependent fluorescent dye tetramethylrhodamine (TMRE, Molecular Probes). 50nM TMRE was added to the cell culture media for 20 minutes at 37˚C and 5% CO_2. Cells were washed, collected, and resuspended in 0.2 ml of PBS. As a control, cells were also treated with the uncoupling agent CCCP at 50 μM. Cells were then analyzed by flow cytometry. Cells were gated for singlets and the mean TMRE fluorescence was obtained using FlowJo analysis software. Mean fluorescence intensity (MFI) values were corrected for CCCP background in each cell type.

Immunoblot analysis. Protein was extracted using cell lysis buffer (Cell Signaling) plus PMSF (600μM) and inhibitor of phosphatases (100x). Protein concentration was quantified using the BCA Protein Assay (Pierce). Protein samples were resolved on SDS polyacrylamide gels (Bio-Rad) and subsequently transferred to nitrocellulose membranes by semi-dry transfer using the Trans-Blot Turbo (Bio-Rad). To determine the expression of mitochondrial proteins in WT-POLG and DN-POLG cells, subunit 70 kDa (SDHA) antibody from MitoSciences (abcam) and COXII antibody from Invitrogen were used. Other antibodies used in this study were: anti-AMPK and anti-p-AMPK from Cell Signaling and anti-ATPIF1 from Sigma. To determine levels of HIF-1α (BD Transduction Laboratories, Clone 54/HIF-1), cells untreated or treated with doxycycline for 6 and 9 days cells were incubated in 1.5% O_2 for 4 hours. As a control, cells were treated with DMOG (1mM, Sigma) or kept in normoxia. β-actin and α-tubulin (Sigma)

were used as loading controls for all protein blots described above. To assess changes in epigenetics marks the following antibodies were used: anti-Histone H3K27ac and anti-Histone H3K14ac from Active Motif, anti-Histone H3K18ac, anti-Histone H3K9ac, anti-Histone H3 (tri methyl K4) and anti-Histone H3 (tri methyl K9) from Abcam, anti-Histone H3 (tri methyl K27) and anti-Histone 3 from Millipore. Anti-rabbit 800CW and anti-mouse 680RD from Licor were used as secondary antibodies. Image Studio Lite version 3.1 (Licor) was used for analysis and quantification of protein levels.

Determination of mitochondrial DNA (mtDNA) content. Genomic DNA was extracted from cells using QIAamp DNA Mini Kit from Qiagen. The relative concentrations of DNA were analyzed by quantitative PCR on a Biorad CFX384 Touch Real-Time PCR Detection System using iQ SYBR Green Supermix (Bio-rad) and the following primer sequences: MTND1-F, CACTGCGAGCAGTAGCCCAA; MTND1-R, GGGTTCGATTCTCATAGTCC; MTCOXII-F, CTGAACCTACGAGTACACCG; MTCOXII-R, TTAATTCTAGGACGATGGGC; SDHA-F, TCCACTACATGACGGAGCAG; SDHA-R, CCATCTTCAGTTCTGCTAAACG; b-actin-F TCCACCTTCCAGCAGATGTG; b-Actin-R, GCATTTGCGGTGGACGAT.

Metabolic assays. Metabolomics of DN-POLG-GFP/BFP and DN-POLG-AOX/NDI1 cells were conducted at Metabolon (Durham, N.C.). To determine glucose and glutamine uptake, 50,000 cells at days 0, 3, 6 and 9 of doxycycline treatment were plated onto PM-M2 plates (Biolog) in Biolog IF-M1 media supplemented with 5% FCS and 0.3 mM L-glutamine. Biolog MB redox dye was added and dye reduction was measured at 590 nm absorbance. Kinetic background values were subtracted and data was normalized to untreated cells. For carbon labeling, isotopic labeling was performed in DMEM with 10% dialyzed FBS supplemented with 10 mM D-[U-^{13}C]glucose and 2 mM L-glutamine. After six hours, metabolites were extracted with 50% methanol and analyzed using an Agilent 6970 gas chromatograph and an Agilent 5973 (Santa Clara, CA, USA) mass selective detector. Analysis of ^{13}C enrichment and mass isotopomer distribution was performed as previously described (Mullen et al., 2014).

ROS Measurements. Cells were stained with 5 μM CellROX Deep Red or CellROX Orange reagents (Life Technologies) in normal growth media for 4h at 37˚C and 5% CO_2. Cells were washed with PBS, collected, and resuspended in 0.2 ml of PBS. Mean fluorescence intensity of live cells was then measured at excitation/emission 640/670 (Deep Red) or 552/582 (Orange) and analyzed using FlowJo software.

RNA-seq analysis. HEK293 cells at day 0, 3, 6, and 9 were collected and prepared for next-generation sequencing using the standard Illumina protocol. The reads passing the initial processing were aligned to the human reference genome (hg19; Genome Reference Consortium GRCh37 from February 2009) with TopHat-Fusion function(Kim and Salzberg, 2011). To determine expression level of wild type versus mutant mitochondrial polymerase, read counts at codon 1135 containing the wild-type sequence (GAC) were compared to read counts containing the mutated sequence (GCG).

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