Supplemental Material

Expanded Materials and Methods

Animals

All protocols were approved by Temple University Lewis Katz School of Medicine Institutional Animal Care and Use Committee. Male and female mice of 9-12 weeks old were used in this study. C57BL/6J mice were purchased from Jackson Laboratory. For MADM mice: Myh6-merCremer (Stock # 005657), and MADM-ML-11$^{GT/TG}$ (Stock# 030578) strains were obtained from The Jackson Laboratory. Mice were kept on a mixed C57BL/6:129SVJ background. Generation and genotyping of these lines has been described previously[61, 62]. Animals were randomly divided into 2 groups: normoxia controls and hypoxia. C57BL/6J mice also included a food restricted group where mice were fed the same amount of food that the hypoxia mice consumed. All mice were fed normal chow.

Hypoxia Chamber

Two-person Vinyl Hypoxic Glove Box (Coy Laboratory) was used for this hypoxia study. The hypoxia chamber was equipped with an automatic purge airlock, automatic/active animal waste filtration system with a carbon dioxide sensor, oxygen control system with an oxygen sensor, nitrogen and oxygen gas regulator, dehumidifier, heated fans, and sleeves with gloves. Activated carbon and carbolime (Coy Laboratory) were used in the animal filtration system to cleanse the air of carbon dioxide and waste when carbon dioxide levels reached above 0.4%. For hypoxia, oxygen was decreased by 1% per day from 20.9% to 7% oxygen. Mice remained at 7% oxygen for an additional 2 weeks and

then placed back in normoxia for terminal echocardiography and euthanized to harvest hearts and tissues. Mice cages had lids with metal/wire bars to allow oxygen to flow into the cages. Mice cages were changed twice a week by placing new cages in the hypoxia chamber through the automatic purge airlock compartment, transferring mice from the old cages to the new cages using the attached sleeves with gloves, and old cages were removed from the hypoxia chamber through the airlock compartment. Water bottles were replaced each week.

Drug administration

Osmotic minipumps (Mini-Osmotic Pump Model 2004, 28 Days delivery, Alzet 0000298) filled with 5-ethynyl-2'-deoxyuridine (EdU; 15 mg/kg/day, Life Technologies E10187) were implanted subcutaneously (SQ) in mice one day before mice were placed in the hypoxia chamber. EdU was dissolved in a 1:1 ratio of DMSO and ddH20. EdU was continuously delivered to the mice for 28 days via the minipump. Mice tissues were harvested at the end of the 4-week study. Tamoxifen (TM) (Sigma) was prepared by dissolving in corn oil (Sigma) to a concentration of 5 mg/mL. TM was injected intraperitoneally using a tuberculin syringe and a 25-gauge needle. Mice were given 50 ug/gram body weight per injection. Normoxic and hypoxic MADM$^{Myh6\text{-}MerCreMer}$ mice received an injection of tamoxifen intraperitoneally once every 3 days for a total of 5 injections during the last 2-week timeframe that mice were at 7% oxygen.

Echocardiography

Mice were placed under anesthesia using 1.5% isoflurane and underwent echocardiography at baseline (1 day before mice were placed in the hypoxia chamber) and at the end of the study; Hypoxic mice were acutely placed in normoxia for terminal

echocardiography then immediately tissues were harvested. Transthoracic Echocardiography was performed to measure cardiac function using the Vevo2100 Ultrasound system (VisualSonics, Toronto, Canada). Images were obtained in the short-axis B mode and M mode at the level of the mid-papillary muscles for analysis of systolic function and chamber dimensions. A full parasternal long-axis view of the heart with the proper landmarks were obtained and M-mode images were collected for right ventricular dimensions as previously described[63, 64]. Length of left ventricle was measured with Simpson's measurements. The anterior wall of the right ventricle was measured by generic depth tool in M-mode. Diastolic function was measured using pulsed Doppler and tissue Doppler imaging (TDI). Analysis was performed offline using VevoLab v3.2.5 (VisualSonics).

Histology and Immunostaining

At terminal, mice were weighed and anesthetized with inhaled isoflurane (Butler Shein Animal Health, Dublin, Ohio). Tissues including the heart and intestines were collected, rinsed with cold PBS, blotted dry, and weighed. Tissues were either snap frozen in liquid nitrogen or fixed with 10% Formalin (heart) or 4% paraformaldehyde (intestines).

Normoxic and hypoxic hearts from C57BL/6J mice were fixed in 10% Formalin, embedded in paraffin, and tissue blocks were sent to AML Laboratories (St. Augustine, FL) for sectioning and slides preparation. Picro-Sirius Red (PSR) staining was performed using a kit (Abcam, ab150681) to measure interstitial fibrosis and images were acquired using a Nikon Eclipse Ti Confocal microscope with DS-Ri2 light camera. Analysis of PSR staining was completed using the color threshold tool from NIH ImageJ software. Cardiomyocyte cross-sectional area was measured by Wheat Germ Agglutinin (WGA,

Life Technologies W11261) staining. Images were taken using Nikon Eclipse Ti Confocal microscope and analyzed using ImageJ. All immunohistochemistry and immunofluorescence experiments included a negative control where a tissue section did not receive primary antibody but was incubated with secondary antibody.

To test the efficacy of the 28-day EdU minipumps, intestines of the mice were fixed in 4% paraformaldehyde, embedded in OCT compound, and sectioned by cryosection. Intestine slides were stained with EdU and WGA after 28 days to detect EdU+ cells. EdU staining was performed using Click-IT EdU Cell Proliferation Kit for Imaging (Life Technologies, C10337) following the manufacturer's protocol. For myocyte proliferation measurements, heart sections were stained with α-sarcomeric actin (Sigma, A2172), WGA (Life Technologies W32466), and either EdU, Ki67 (Abcam ab15580), or pH3 (Millipore 06-570). Cell nuclei were stained with 4',6-diamidino-2phenylinodole (DAPI, Millipore 268298). The heart was collected during terminal study and cut horizontally into 4 sections from base to apex. Slides were made and used for histological analysis. Images were taken for each of the 4 sections of the heart. In total, images of the atria (4-5 images), left ventricle (LV, 12-20 images), and right ventricle (RV, 12-20 images) were taken for each mouse heart using a 20X objective. The images included pictures of the endocardium, myocardium, and epicardium of the Atria, LV, and RV. NIS Confocal analysis software was used to analyze the images.

For angiogenesis measurements, heart slides were stained with CD31 (R&D Systems AF3628) and EdU to quantify capillary density and size, and endothelial cell proliferation. Heart sections were stained with α-smooth muscle actin (αSMA, Abcam ab5694) to label fibroblasts. CD45 (R&D Systems AF114) and EdU staining labeled proliferative immune

cells in the heart. Apoptotic cells were detected by Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining using DeadEnd Fluorometric TUNEL system (G3250, Promega; Madison, WI). Analysis was done using Object Count tool on NIS confocal analysis software.

MADM$^{Myh6\text{-}MerCreMer}$ mice hearts were fixed in 0.05% paraformaldehyde (PFA) in PBS solution, embedded in Tissue-Tek O.C.T. Compound (Sakura 4583), and slides were prepared by cryosectioning tissue. Slides were stained with WGA and DAPI; green fluorescent protein (GFP) and red fluorescent protein (RFP) were auto-fluorescent from the tamoxifen-induced Cre-loxP recombination[24]. Images were taken using the Nikon Eclipse Ti Confocal microscope and analyzed using NIH ImageJ software for the total number of yellow cardiomyocytes (both GFP+ and RFP+), and single labeled GFP+ and RFP+ cardiomyocytes.

All representative images chosen were the best images that represent the mean of the data.

Cardiomyocyte Isolation

Myocytes were isolated from the LV and RV of the heart as previously described[65-67]. Briefly, the aorta of the heart was cannulated and perfused with collagenase type II (Worthington LS004177) to enzymatically digest the heart. When the myocardium was softened, the atria were removed, and the ventricles were separated and placed in separate dishes. The LV and RV of the heart were mechanically dissociated to yield single cardiomyocytes. Numbers of LV-/RV-isolated single cardiomyocytes were counted with hemocytometer respectively. The cell solution was replaced with a solution containing bovine serum albumin (BSA) and calcium. Myocytes were plated on laminin coated

dishes and placed in a 37C incubator for 30 minutes to allow cells to adhere to the dish. Then, dishes containing the myocytes were washed in Normal Tyrode's solution and fixed with 4% paraformaldehyde in PBS. Dishes were handled very carefully to reduce myocyte loss. For staining, myocytes were permeabilized using a Triton X (Sigma T8787) diluted solution and dishes were stained with EdU and DAPI. Images were taken on the Nikon Eclipse Ti Confocal microscope. At least 100 isolated cardiomyocytes from the LV and RV of each mouse were analyzed. NIS Confocal analysis software and ImageJ were used to analyze the images.

Liquid Chromatography-Mass Spectrometry (LC-MS)

Liquid chromatography-Mass spectrometry was performed on normoxic and hypoxic plasma samples of MADM$^{Myh6\text{-}MerCreMer}$ mice that were collected at the end of the study (2-3 days following the last tamoxifen injection), to measure Tamoxifen (TAM) and its metabolite, 4-hydroxytamoxifen (4OHTAM). The metabolomics analysis was performed at The Wistar Institute Proteomics and Metabolomics Shared Resource on a Thermo Q-Exactive HF-X mass spectrometer purchased with NIH grant S10 OD023586.

Tamoxifen (TAM) and (Z)-4-Hydroxytamoxifen (4OHTAM) were purchased from Toronto Research Chemicals (catalog# T006077 and H954757, respectively), and 1 mg/ml stock solutions were made in methanol and stored at -20 °C. Mouse plasma was deproteinated and extracted with 10-fold volume ice-cold 80% methanol, 0.1% formic acid that was spiked with 1.5 µM heavy-labeled amino acid internal standard mix (MSK-A2-1.2; Cambridge Isotope Laboratories). Extracts were stored at -80 °C prior to analysis. Matrix-matched calibration standards ranging from 0.008 to 500 ng/ml were generated from TAM and 4OHTAM in a pool of plasma extracts from mice that were not treated with TAM. LC-

MS analysis was performed on a Vanquish Horizon UHPLC in-line with a Thermo Scientific Q-Exactive HF-X mass spectrometer with HESI II probe. Chromatographic separation used a Synergi Polar-RP column (100 × 2 mm, 4 µm particle size, Phenomenex) at 45 °C with a flow rate of 0.3 ml/min. Solvent A was 0.1% formic acid, and solvent B was acetonitrile, 0.1% formic acid. The gradient was 5% to 95% B over 10 min, hold at 95% B for 4 min, 95 to 5% B over 0.1 min, and hold at 5% B for 5.9 min. The autosampler was held at 4 °C, and 2 µl of each calibration standard or sample was injected. The MS method was performed in positive polarity and used full MS scans from 72-260 m/z for 0-3 min (for heavy standards) followed by selected ion monitoring (SIM) scans from 350-400 m/z for 3-20 min (for TAM and 4OHTAM). Scans were acquired at 120,000 resolutions with 3e6 automatic gain control (AGC) and 100 ms injection time (IT). Other relevant MS parameters: sheath gas flow rate, 10; spray voltage, 4 kV; capillary temperature, 320 °C; and funnel RF level, 40.

Data analysis was performed using TraceFinder 4.1 (Thermo Fisher Scientific). Compounds were detected by the ICIS detection algorithm with default parameters based on the calculated mass of [M+H]+1 adducts with 5 ppm mass error and retention time match of analytical standards. Quantitation used integrated peak area. External calibration curves were generated using quadratic curve-fit with 1/x2 weighting (R2 = 0.9924 and 0.9912 for TAM and 4OHTAM, respectively). Compound stability during the course of the analysis was assessed by analyzing 2-3 calibration standards at the beginning and end of the sample sequence (RSD < 10%). Reproducibility of extraction was verified by recovery of the internal standards (RSD < 20%).

Real-time polymerase chain reaction (RT-PCR)

Hearts were collected, rinsed with PBS, and snap frozen in liquid nitrogen during harvest. Total RNA was extracted from frozen cardiac tissue using miRNeasy Mini Kit (Qiagen 217004) following the manufacturer's instructions. Then samples were digested with DNase I (Invitrogen 18068) to remove genomic DNA. SuperScript III first strand synthesis system (Invitrogen 18080) was used to synthesize DNA. RT-PCR was performed using the Quantifast Sybrgreen PCR kit (Qiagen) and the StepOnePlus Real-Time PCR system (Applied Biosystems). Ct values were normalized to beta-2 microglobulin (β2M), and fold changes were calculated with respect to normoxia control mice. The following primer sets were used (forward, reverse): β2M 5'- ATGTGAGGCGGGTGGAACTG, 5'- CTCGGTGACCCTGGTCTTTCTG; Periostin (Postn) 5'- TATGCTCTGCTGCTGCTGTT, 5'- TTTCTTCCCGCAGATAGCAC; Col1A1 5'- GCCAAGAAGACATCCCTGAA, 5'- GCCATTGTGGCAGATACAGA; Col3A1 5'- AGAGGCTTTGATGGACGCAA, 5'- CCACCAGGACTGCCGTTATT.

RNA Sequencing

Standard RNA sequencing of extracted RNA from frozen hearts (n=3 per group) of male (normoxia) controls (MC), male hypoxia (MH), female controls (FC), and female hypoxia (FH) were performed by Fox Chase Cancer Center (Philadelphia, PA) with an average of 30 million reads per sample. The reagent used for RNA sequencing was NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina (Cat #E4720L). Stranded mRNA-seq library: 100-1000ng total RNAs from each sample were used to make library according to the product guide of stranded mRNA library kit. In short, mRNAs were enriched twice via poly-T based RNA purification beads and subjected to fragmentation at 94 degrees for 15 min via divalent cation method. The 1st strand cDNA was synthesized by reverse

transcriptase and random primers at 42 degrees for 15 mins, followed by 2nd strand synthesis at 16 degrees for 1hr. During second strand synthesis, the dUTP was used to replace dTTP, thereby the second strand was quenched during amplification. A single 'A' nucleotide is added to the 3' ends of the blunt fragments at 37 degrees for 30 min. Adapters with illuminaP5, P7 sequences as well as indices were ligated to the cDNA fragment at 30 degrees for 10 min. After SPRIselect beads (Beckman Coulter, Cat# B23318) purification, a 15-cycle PCR reaction was used to enrich the fragments. PCR was set at 98 degrees for 10 sec, 60 degrees for 30 sec and extended at 72 degrees for 30 sec. Libraries were again purified using SPRIselect beads, had a quality check on Agilent 2100 bioanalyzer (serial # DE34903146) using Agilent high sensitive DNA kit (Cat# 5067-4626), and quantified with Qubit 3.0 fluorometer (ThermoFisher Scientific, Cat#Q33216) using Qubit 1x dsDNA HS assay kit (Cat#Q33230). Sample libraries were subsequently pooled and loaded to the Hiseq 2500 (Illumina, serial number SN930). Paired end reads at 75bp were generated by using Nextseq 2000 P2 reagent kit (Illumina, Cat# 20046811). Fastq files were obtained at Illumina base space (https://basespace.illumina.com).

Raw data quality was evaluated with FastQC, reads were mapped onto Mus musculus GRCm39 reference genome with Salmon Software. Genome is available on GENCODE. Quant files were imported, and transcript-level abundance was estimated with R package Tximport, 39210 genes were mapped. QC was performed, genes with counts per million (CPM) ≥0.5 were kept. 15208 genes out of 39210 genes passed filter. Genes were identified on mapped reads followed by downstream differential expression analysis using R package DESeq2 performed on the input matrix by DESeq function[68]. Differentially

expressed genes (DEGs) that were considered either up- or down- regulated required to have a false discover rate (FDR) cutoff ≤0.1 and fold change ≥2. The output was subjected to res function that created a result file detailing log2 fold-change (LFC) and adjusted p-value for each specific sample: sample comparison. With regularized logarithm (rlog) function, count data was transformed for visualization on a log2 scale. Gene counts, log2 fold change and adjusted p-values between comparisons of all 15208 genes were provided in Data Set S2 (Diff_expression_all_comparisons.csv).

Gene set enrichment analysis (GSEA) was performed with ClusterProfiler package based on gene ontology biological process or molecular function, all filter-passed genes with their log2FC were provided for the analysis. Significant enriched gene sets were determined with a cutoff p-value 0.05. Top activated and suppressed gene sets were graphed with R package ggplot. Hierarchical clustering of genes in targeted GO terms was performed with seaborn.clustermap (0.10.0) module in Python.

The RNA-sequencing data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus[69] and are accessible through GEO Series accession number GSE221168 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE221168).

Statistical Analysis and Blinding

All data was analyzed using GraphPad Prism software (Version 9.3.1, GraphPad Software Inc., San Diego, CA). Data are shown as mean±SD and a p-value of $\leq$ 0.05 was used to determine significance for all statistical analysis. The distributions of all continuous variables were tested for normality assumptions using the Shapiro-Wilk normality test using GraphPad Prism. Weight measurements and results of immunostaining were analyzed using unpaired 2-sample t-test or the Mann-Whitney test

depending on the distribution of the data to compare single measurements within the two treatment groups of normoxia and hypoxia mice. Comparison between multiple groups were performed by one-way ANOVA, followed by Tukey's post-hoc multiple comparisons test. For echocardiography parameters, two-way ANOVA followed by Turkey's or Sidak's multiple comparisons test was used to analyze treatment group differences at each time point and changes between baseline and terminal time points within each treatment group. Two-sided testing was used for all statistical tests. Investigators of this study were blinded to the treatment group of the animals, following tissue harvest. Investigators were unblinded after data analysis was completed.

Data Availability

The data, methods used in the analysis, and materials used to conduct the research will be made available to any researcher for purposes of reproducing the results. Data of standard RNA-sequencing have been deposited in NCBI's Gene Expression Omnibus[69] and are accessible through GEO Series accession number GSE221168 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE221168).

Supplemental Figures and Legends

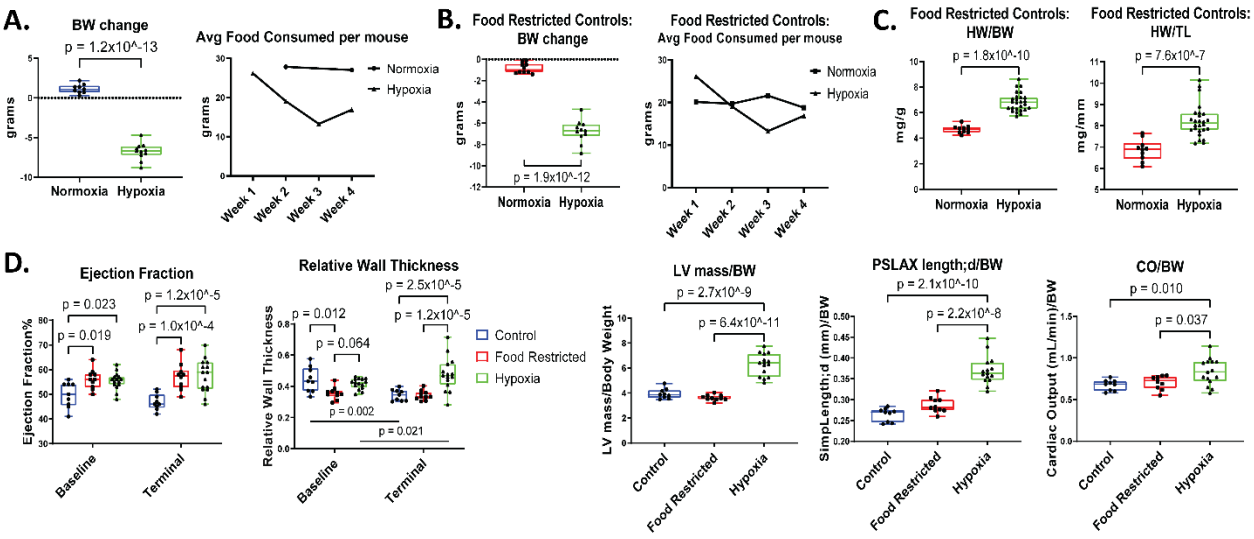
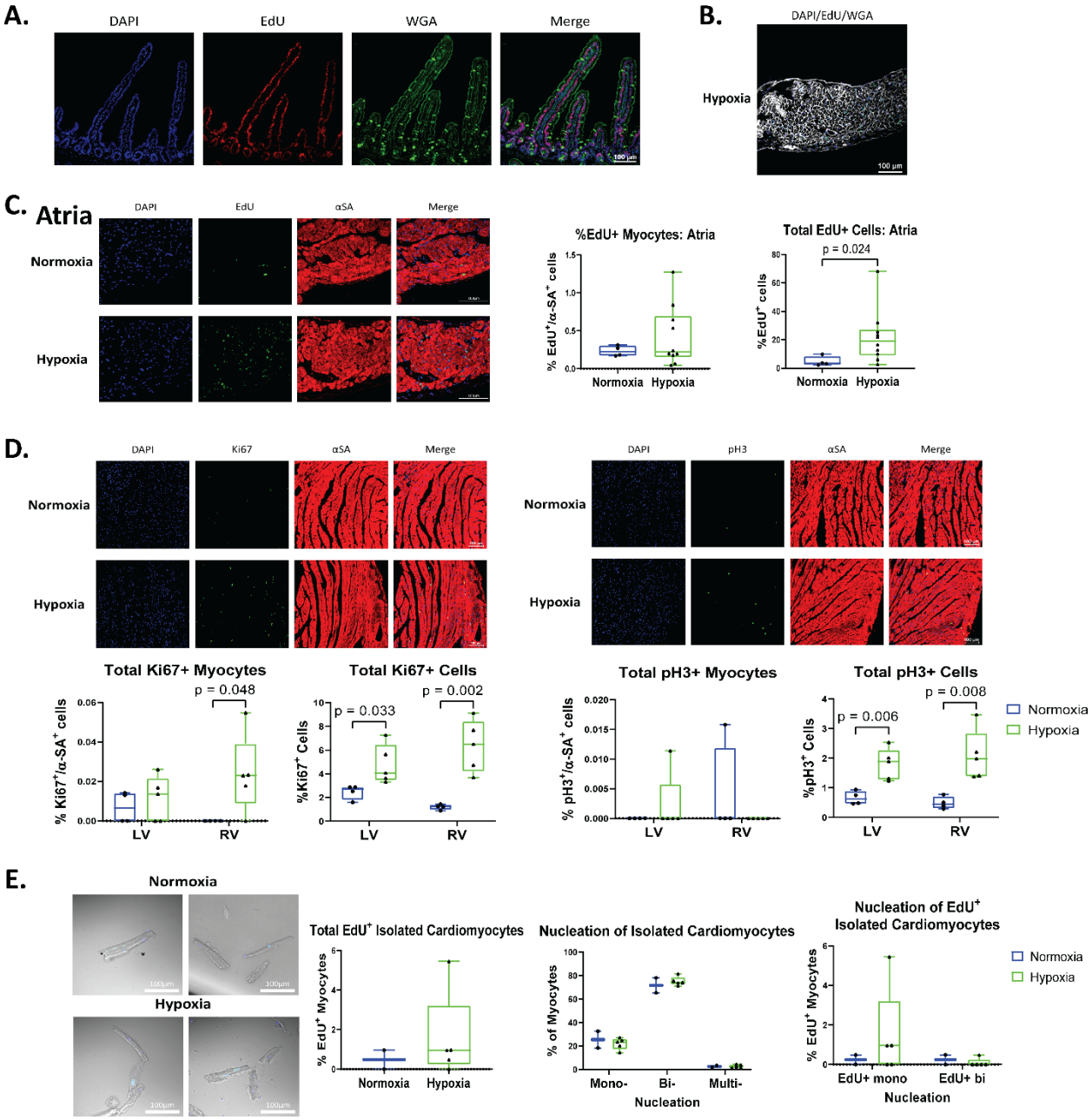


Figure S1. Hypoxia induces cardiac hypertrophy and maintains cardiac function when compared to control mice on a food restricted diet.

A: Change in body weight (BW) of normoxia controls and hypoxia mice from baseline to terminal study. The average amount of food each mouse consumed was weighed weekly and recorded (normoxia n=9, hypoxia n=11). B: Body weight change and weekly food intake for each mouse was calculated from the beginning to end of study for normoxia controls on a food restricted diet and hypoxia mice (n=10-11). C: Heart weight to body weight (HW/BW) and heart weight to tibia length (HW/TL) of food restricted controls and mice at low oxygen (food restricted normoxia n=10, hypoxia n=26). D: Echocardiography was performed on normoxia controls, food restricted normoxia controls, and hypoxia mice to measure ejection fraction and relative wall thickness at baseline and terminal. Left ventricular (LV) mass, parasternal long axis (PSLAX) length of the LV, and cardiac output (CO) relative to body weight was also determined (n=9-15). Data represented as mean±SD.



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271 **Figure S2. Efficacy of EdU minipumps and additional evidence of cardiomyocyte**
272 **proliferation.**

273 A: EdU minipumps were placed in control mice for 28 days and intestinal tissue was
274 collected at harvest. Representative images of murine intestinal tissue stained with WGA
275 (green, labels membrane of cells), EdU (red, cells in DNA synthesis), and DAPI (blue,
276 nuclei). B: For analysis of total EdU+ myocytes, mouse heart sections were stained with

WGA (white), EdU (green), and DAPI (blue) to quantify proliferating myocytes. C: Atrial sections of the heart were labeled with alpha-sarcomeric actin (αSA, red, stains cardiomyocytes), EdU (green), and DAPI (blue). Quantification of EdU+ cardiomyocytes and total EdU+ cells in the Atria of normoxia and hypoxia mice (normoxia n=4, hypoxia n=10). D: Representative images of cardiac tissues stained with αSA (red), DAPI (blue), and either Ki67 (green, cells in DNA synthesis) or pH3 (green, cells in mitosis). Quantification of total Ki67+ or total pH3+ cardiomyocytes and cells in LV and RV sections of the heart (normoxia n=4, hypoxia n=5) E: Isolated ventricular cardiomyocytes from normoxia and hypoxia mice were labeled with EdU (green) and DAPI (blue). Total EdU+ myocytes, nucleation of total myocytes, and nucleation of EdU+ myocytes was quantified. Data represented as mean±SD.

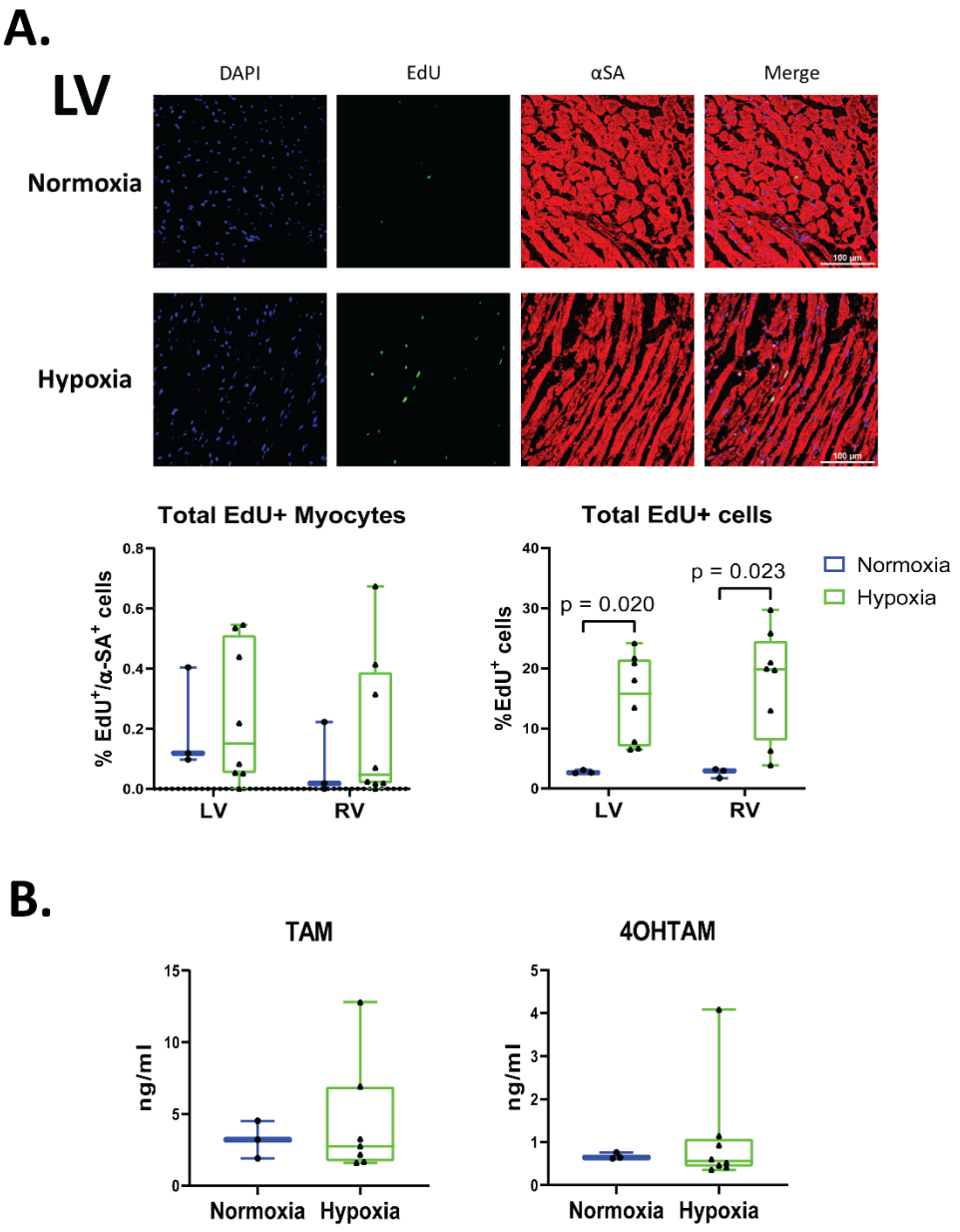


Figure S3. Hypoxia stimulates DNA synthesis in cardiomyocytes and non-myocyte cells and reduces metabolism of tamoxifen in MADM mice.

A: Representative confocal images of left ventricular (LV) sections of hearts from normoxic and hypoxic MADM$^{Myh6\text{-}MerCreMer}$ mice were labeled with αSA (red, cardiomyocytes), EdU (green, cells in DNA synthesis), and DAPI (blue, nuclei), following harvest. Total EdU+ myocytes and EdU+ cells were quantified in LV and right ventricular (RV) heart sections (normoxia n=3, hypoxia n=8). B: Absolute levels of Tamoxifen (TAM)

and its metabolite, 4-hydroxytamoxifen (4OHTAM) were measured from plasma of normoxic and hypoxic MADM$^{Myh6\text{-}MerCreMer}$ mice. Data represented as mean±SD.

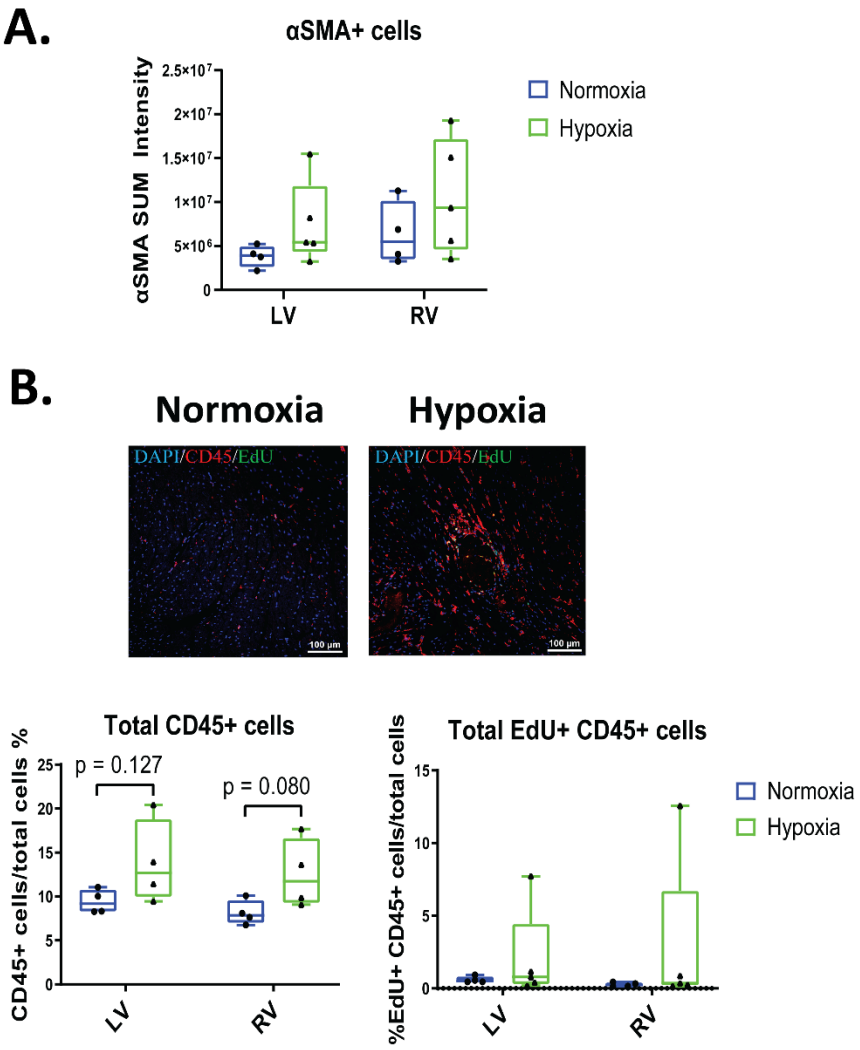


Figure S4. Increase of fibroblasts and immune cells in hearts under chronic hypoxemia.

A: Normoxic and hypoxic hearts were stained with alpha-smooth muscle actin (αSMA) and SUM intensity was quantified in LV and RV myocardium. B: Cardiac ventricles were labeled with CD45 (red, stains immune cells), EdU (green), and DAPI (blue) to analyze total CD45+ cells and total EdU+ CD45+ cells in normoxia and hypoxia mice (n=4-5). Data represented as mean±SD.

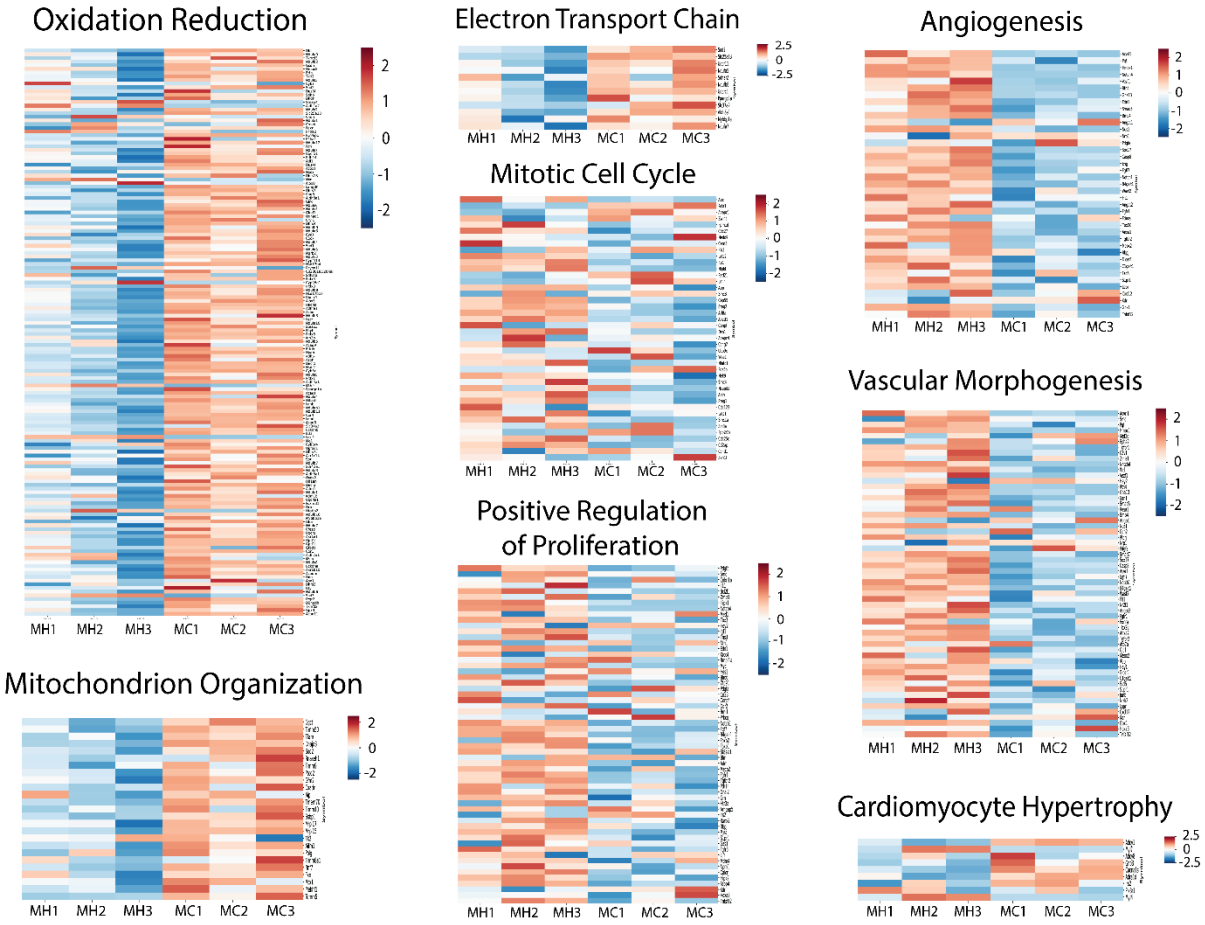


Figure S5. Heat maps of several biological processes in normoxic and hypoxic male hearts.

Analysis of RNA sequencing on ventricles of male hearts revealed biological processes that are differentially regulated in hypoxia (MH) mice and normoxia controls (MC). Upregulated and down regulated genes are shown as red and blue, respectively (n=3).

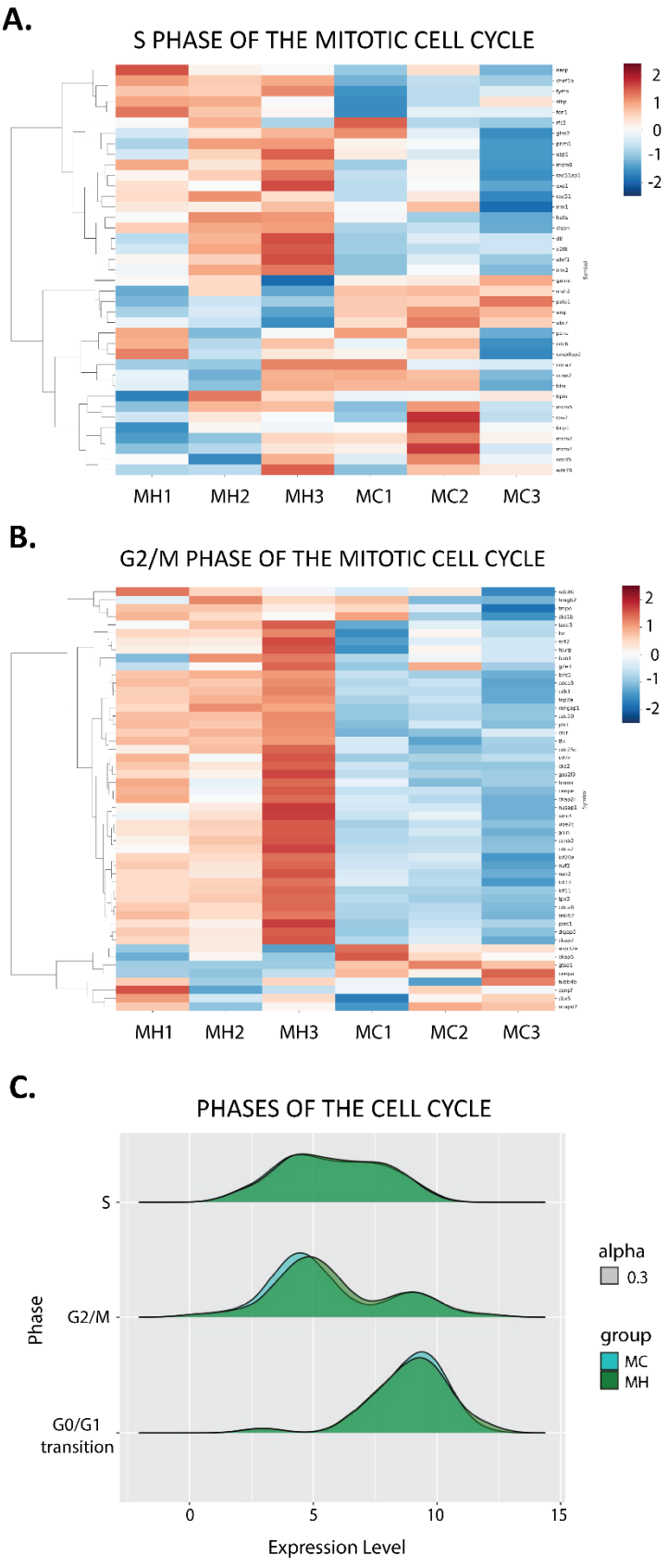
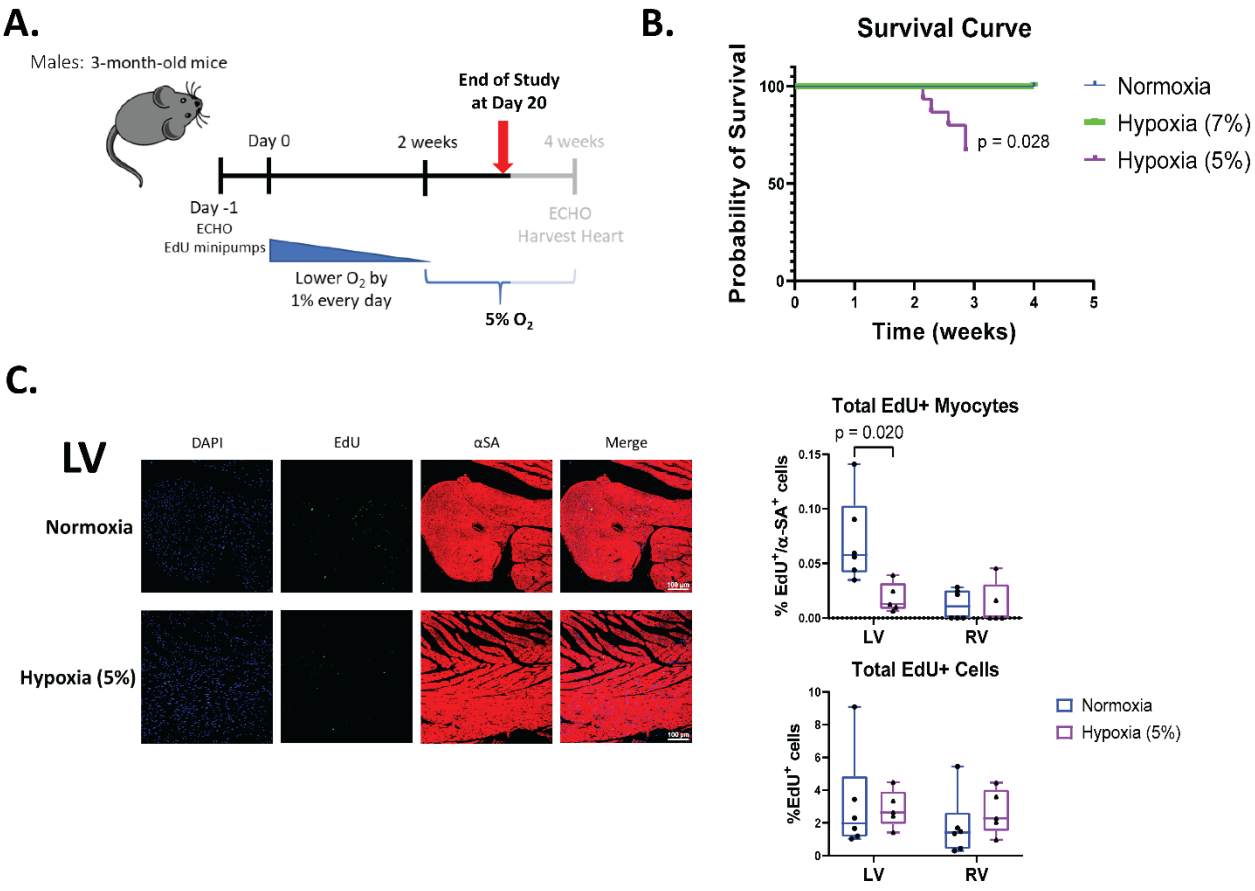

A.
S PHASE OF THE MITOTIC CELL CYCLE
2
1
0
-1
-2
MH1
MH2
MH3
MC1
MC2
MC3
B.
G2/M PHASE OF THE MITOTIC CELL CYCLE
2
1
0
-1
-2
MH1
MH2
MH3
MC1
MC2
MC3
C.
PHASES OF THE CELL CYCLE
S
G2/M
G0/G1
transition
Phase
alpha
0.3
group
MC
MH
0
5
10
15
Expression Level

Figure S6. Hypoxia alters the mitotic cell cycle in male hearts.

A and B: Analysis of RNA sequencing on ventricles of male hearts revealed heat maps of S phase and G2/M phase of the mitotic cell cycle that are differentially regulated in hypoxia (MH) mice and normoxia controls (MC). Upregulated and down regulated genes are shown as red and blue, respectively (n=3). C: Graph of the expression level of genes in S phase, G2/M phases, and G0 to G1 transition of the mitotic cell cycle. The x-axis represents the average expression level of each gene from each group. The y-axis represents the number of genes that fall into the range of that expression level. Hypoxia group (MH) is labeled green, and normoxia group (MC) is labeled blue.



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383 **Figure S7. Severe hypoxemia (5% oxygen) causes death and inhibits cell**
384 **proliferation in male mice.**

385 A: Schematic of severe hypoxia treatment with gradual reduction in oxygen to reach 5%
386 oxygen. B: Survival curve of male mice exposed to normoxia (20.9% oxygen), hypoxia
387 (7% oxygen), and severe hypoxia (5% oxygen) during the study (n=6-15). C: Cardiac
388 tissue was stained with alpha-sarcomeric actin (αSA, red, labels cardiomyocytes), EdU
389 (green, cells undergoing DNA synthesis), and DAPI (blue, nuclei) to measure cell
390 proliferation. Total EdU+ myocytes and EdU+ cells were quantified in the left ventricle
391 (LV) and right ventricle (RV) of the heart (n=5-6). Data represented as mean±SD.

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393