SUPPLEMENTAL MATERIAL

EXPANDED METHODS

Murine Models

The generation of the Mybpc3 null model (Mybpc3^{tm1d}) was previously described.[20] The following murine strains were obtained from the Jackson laboratory: Mdm2$^{tm2.1Glo}$ (031614), Hif1a^{tm3Rsjo} (007561), Hif2/Epas1^{tm1Mcs} (008407), Myh6:Cre (011038**)**, and Myh6:MerCreMer (005657). All of the mice obtained from Jackson labs were bred to a C57BL/6J genetic background for at least six generations.

The Myh6^{R404Q} mouse line was generated by first identifying Cas 9 targets using CRISPOR[61] and the guide with the lowest potential off target binding sites was chosen **(Table S1).** The single guide RNA was synthesized as previously described.[62] A mutant donor template DNA encoding the R404Q mutation was created with the specific missense mutation necessary to introduce the R404Q amino acid change and silent DNA substitutions were introduced to create diagnostic restriction sites and to block subsequent rounds of CRISPR/Cas9 target sequence cutting (**Table S1**). The guide RNA and Myh6-R404Q donor template successfully created a high rate of gene edited founders but they were all homozygote or compound heterozygote for the Myh6 R404Q mutation and/or Myh6 indels and subsequently died within 7-12 days of birth. Therefore, we created a wild type donor template DNA with a CRISPR blocking mutation (**Table S1**) which could be co-injected with the Myh6-R404Q mutant template. Fertilized embryos (C57BL/6J, Jackson Laboratory) produced by natural mating were microinjected in the pronuclei stage with a mixture of 0.66 μM EnGen Cas9 protein (New England Biolabs, Cat.No. M0646T), the Cas9 guide RNA (42.5 ng/μl) and two DNA oligonucleotides, Myh6-R404Q and Myh6-WT (0.5 μM each, synthesized by Integrated DNA Technologies). Injected zygotes were cultured overnight, allowed to develop to the 2-cell stage and then were transferred to the oviducts of pseudopregnant CD1 female surrogates. The founder mice underwent sequencing to confirm the accuracy of Myh6

allele gene editing and to identify if off-target mutations were introduced. Founder mice were backcrossed to wild type C57BL/6J animals to create heterozygote Myh6$^{R404Q/WT}$ mice and were further backcrossed for 5 generations before utilization in the current study **(Figure S6A+B)**.

mRNA Isolation and Quantitative RT-PCR

Euthanasia and heart mass assessments were performed as described previously.[20] Tissues for RNA analyses were flash-frozen in liquid nitrogen and placed in -80°C. RNA was extracted from LV heart tissue from post-natal (P) day 7 mouse pups using TRIzol reagent (Invitrogen) after column purification (Zymogen). cDNA was generated from RNA using QuantiTect reverse transcription kit (Qiagen). Gene-specific primers were designed and SYBR Green reagent (Applied Biosystems) was used to perform the PCR (QuantStudio). Two technical replicates of each sample were prepared. Desired genes were normalized to endogenous housekeeping gene Rpl32 using 2 – ΔΔCt method. The primer sequences utilized for RT-PCR analysis are listed in **Table S2.**

Immunohistochemistry and Immunofluorescence of Heart Sections

Hematoxylin & Eosin (H+E) staining of myocardial tissue: Hearts were embedded in paraffin or optimal cutting temperature (OCT) compound. 5 or 10 μm heart cross sections were obtained from the apex to the base of the heart and H&E staining was then performed.

Immunohistochemistry of myocardial tissue: Cryosections were cut from OCT embedded myocardial tissues at 5-10 μm. The sections were then fixed for 15 minutes with 4% paraformaldehyde (PFA) and washed with phosphate buffered saline (PBS). They were then permeabilized with 0.2% Triton X-100 and again with PBS. Sections were then incubated with 1% Bovine serum albumin (BSA) in PBS for 1 hour at room temperature. Solo or double primary antibody overnight incubation with HIF1α (1:500, Novus Biologicals, nb100-479), HIF2α (1;500, Novus Biologicals, nb100-122), PCM-1 (1:500, Sigma-Aldrich, HPA023370), MDM2 (1:500,

Novus Biologicals, NB100-2736), VHL (1:500, Invitrogen, PA5-27322), CD31 (1:100, Invitrogen, 14-0311-82), TIE2 (1:50, R&D Systems, AF762), NG2 (1:1000, Miltenyi Biotec, 130-097-455), CX40 (1:100, Bicellscientific, 00405) and p-Ser139 Histone h2ax (1:500, γh2ax, Millipore Sigma, 05-636-I) at 4°C was performed. After PBS washes, sections were placed in dark incubation for one hour at room temperature with the following fluorescent secondary antibodies: goat anti-rabbit Alexa Fluor 594 (1:500, A-11005), goat anti-mouse Alexa Fluor 488 (1:500, A-10667), donkey anti-goat Alexa Fluor 488 (1:500, A-11055) and/or donkey anti-mouse Alexa Fluor 594 (1:500, A-21203) (Thermo Fisher Scientific). After PBS rinses, sections were mounted with ProLong Gold Antifade with DAPI (Thermo Fisher Scientific) and images were acquired at 10x, 20x, 40x or 100x magnification on a fluorescence microscope (Zeiss Axioplan or Nikon Eclipse TI2). Images were analyzed using ImageJ (http://imagej.nih.gov/ij/) and protein expression was calculated by manual counting and/or fluorescence intensity quantification (as noted in the figure legends).

Myocardial tissue capillary density quantification: Immunohistochemistry was performed with CD31 or TIE2 or NG2 antibodies as described above. The sections were then incubated for 10 minutes with Texas Red or Oregon Green 488- conjugated WGA (Thermo Fisher Scientific, W21405/W6748) diluted 1:200 in PBS. After PBS rinses, sections were mounted with ProlongGold Antifade with DAPI. Images with cardiomyocytes in cross-section were taken from the LV free-wall regions at equal intensities under 40x magnification. To obtain capillary count, capillary marker channel was first merged with WGA channel using ImageJ. Non-specific background was eliminated by equally adjusting minimum and maximum display range values so that no fluorescence was localized inside the cardiomyocyte cytoplasm. Capillary marker channel was split from WGA channel, and capillaries were manually counted using cell counter plugin in ImageJ. Vessels less than 10um in size were classified as capillaries and included in the analysis.[63] Cardiomyocytes were counted from WGA channel and capillary cardiomyocyte ratios were then calculated (**Figure S1A**). Regions where cardiomyocytes and capillaries were not in

cross section were excluded from analysis. CD31 or TIE2 fluorescence intensity was calculated by processing images to eliminate non-specific background as described above in ImageJ and then measuring the total fluorescence intensity per field.

Coronary artery cross-sectional area: Immunohistochemistry was performed with CD31 and CX40 antibodies as described above. Large CD31 positive vessels were confirmed as coronary arteries, and not veins, using CX40 which is a marker for endothelial cells of large arteries. Images for left coronary artery in cross-section were taken at mid ventricular level of the heart at 10x or 20x magnification. Arterial walls were manually traced, and coronary artery cross-sectional area were quantified using Image J software.

Cardiomyocyte DNA damage quantification: Immunohistochemistry was performed with DNA damage response marker γH2AX and PCM1 or CD31 antibodies as described above. Images with cardiomyocyte or endothelial nuclei in cross section were taken from LV free-wall regions at 40x magnification. Nuclear γH2AX fluorescence was determined in cardiomyocytes and endothelial cells using the following equation: corrected nuclear fluorescence = integrated density– (area of nuclei × mean fluorescence of background readings), where integrated density is the fluorescence intensity of the defined region of interest (ROI), area of nuclei is the size of the defined ROI and mean fluorescence background is the average intensity of 5 background ROI.

Tomato Lectin Capillary Staining

Neonatal and adult mice were sedated by 5% isoflurane. A mix of 100 ug of Dylight 488-conjugated tomato lectin (Thermo Fisher Scientific, L32470) in 100 µl volume and 100 µl of PBS (total of 200 µl) was directly injected into the LV cavity in adult mice. A total of 100 ul was injected via retroorbital route in neonatal mice.[64] After waiting for two minutes, the hearts were excised and embedded in OCT and 50 µm sections were obtained, slides were mounted and images were acquired for LV free-wall regions. When immunohistochemistry for CD31 or NG2 was performed

in combination with lectin staining, the sections were fixed using the method for immunohistochemistry described above.

Tissue Hypoxia Assessment

Mice were injected intraperitoneally with 60 mg/kg of pimonidazole (HP2, Hypoxyprobe Inc) and then euthanized 1hr later. The hearts were harvested and embedded in OCT. 5 µm tissue sections were obtained and fixed with 4% PFA. Sections were permeabilized with 0.2% Triton X-100 and incubated with 1% BSA in PBS for 1 hour at room temperature. Sections were then incubated with a fluorescein-conjugated, anti-pimonidazole, mouse IgG1 monoclonal antibody (Hypoxyprobe Inc) for 1 hour at room temperature in darkness. After PBS washes, sections were counterstained and mounted with ProLong Gold Antifade with DAPI (Thermo Fisher Scientific) and allowed to cure for 30 min in the dark. Slides were imaged at 5x, 20x and 40x magnification (Zeiss Axioplan). Images were processed in ImageJ (http://imagej.nih.gov/ij/) to measure green fluorescence intensity. Multiple images were acquired and stitched together to reconstruct the contour of the heart using the Fiji ImageJ plugin for pairwise-stitching.

Coronary Flow Reserve Measurement

Echocardiography and doppler measurement of myocardial blood flow (MBF) was performed using previously described methods.[65,66] In brief, transthoracic echocardiography on anesthetized mice at P25 and P60 was performed using a Vevo 3100 (VisualSonics Inc). Short axis B-mode images were acquired, and color Doppler was used to identify the left coronary artery (LCA) and measure the internal LCA diameter **(Figure S2J)**. Baseline LCA velocity was obtained using doppler **(Figure S2K)** and heart rate was recorded. Mice were then injected with adenosine (0.2 mg/mouse diluted in PBS) retro-orbitally to achieve maximal coronary vasodilation. Maximal hyperemia was achieved after 2 minutes of adenosine injection. Internal LCA diameter and coronary flow velocity were then remeasured, and heart rate was recorded. Velocity time interval (VTI) was then obtained by measuring the area within LCA diastolic flow velocity curve **(Figure**

S2L). Myocardial blood flow (MBF) was then calculated using the formula MBF(ml/min) = ((π/4) x D^2 x VTI x HR) where D is the internal LCA diameter (mm), VTI (velocity time integral) and HR is the heart rate. Myocardial blood flow (MBF) was then indexed to total heart mass by dividing the MBF by heart weight (mg). Coronary flow reserve (CFR) was defined as the ratio of post-adenosine MBF divided by baseline MBF (CFR= $MBF_{post\text{-}adenosine}/MBF_{baseline}$).

Immunoblotting and Antibodies

LV tissue lysates were homogenized in chilled RIPA buffer containing protease and phosphatase inhibitors (Thermo Fisher Scientific, 78430). Proteins were then separated by SDS-PAGE utilizing 10% Criterion TGX precast gel (Bio-Rad) and transferred to 0.45 μm pore size PVDF (Millipore Sigma) or nitrocellulose membrane (Bio-Rad) at 100 V for 60 minutes in 4 degrees. Membranes were blocked for one hour at room temperature with 5% BSA or nonfat dry milk in TBS containing 0.1% Tween-20 (TBST). Blots were incubated with the following primary antibodies: HIF1α (Novus Biologicals, NB100-479 and Figure S5C NB100-105), HIF2α (Novus Biologicals, NB100-122), MDM2 [SMP14 (Novus Biologicals, NB100-2736), Figure S3M Ab-3/4B11 (Sigma-Aldrich, OP143) and 42BC1.11 (Sigma-Aldrich, MABE331)], VHL (Invitrogen, PA5-27322), MYBPC3 (SCBT, sc-137180), K48-linked ubiquitin (CST, 8081), Ubiquitin (SCBT, sc8017), p53 (CST, 2524) or β-actin (CST, 8457) overnight at 4°C. For chemiluminescent immunoblot detection, membranes were incubated with goat anti-rabbit or anti-mouse horseradish peroxidase (HRP)-linked secondary antibodies for 1 hour at room temperature. The membranes were then developed with Clarity ECL Western substrate (Bio-Rad) or ultra-sensitive Supersignal West Femto Substrate (Thermo Scientific). Images were captured under ChemiDoc MP Imager (Bio-Rad) by highlighting saturated pixels to control for overexposure. For fluorescent immunoblot detection, goat anti-mouse 680LT or goat anti-rabbit 800CW IRDye (Licor Biotech) was utilized, and the membrane was then imaged using an Odyssey CLx Infrared imager (Licor Biotech). Densitometric analyses were performed using Image J, Image Lab (Bio-Rad) or Image Studio Lite

software (LI-COR Biosciences). To control for loading differences between samples, β-actin or a total protein stain (Revert LI-COR) was utilized as delineated in the figure legends.

Co-Immunoprecipitation

Protein extraction from LV tissue was carried out using chilled Pierce IP lysis buffer (Thermo Fisher Scientific, 87788) with protease and phosphatase inhibitors. For co-immunoprecipitation, MDM2 antibody (1 ug/ml) was incubated with 100 µl of pre-washed SureBeads Magnetic Beads (Bio-Rad, 161-4013) for 1 hour on a rocking platform. Next, antibody-conjugated beads were collected by magnetization, washed in PBST, and then incubated with 500 ug of cardiac tissue lysate for 2 hours on a rocking platform. Samples were then collected by magnetization and washed a minimum of three times with IP lysis buffer containing protease/phosphatase inhibitors. After removing the supernatant, proteins were eluted with 2X Laemmli sample buffer followed by heating at 95°C for 5 min and western blots for MDM2 and VHL were performed. A measure of 10% of the co-IP homogenate was taken for examination of the input and loading control.

Immunoprecipitation

Protein extraction from LV tissue was carried out using chilled RIPA buffer containing SDS (Thermo 78430) with protease/phosphatase inhibitor (Thermo 78440) and deubiquitinase (DUB) inhibitor PR-619 (Apexbio, A8212, 50 uM). For immunoprecipitation, the following primary antibodies (MDM2 1 ug/ml, HIF1α 1 ug/ml, HIF2α 1 ug/ml, VHL 1 ug/ml) were incubated as described section in co-immunoprecipitation. Western blots for K48-linked poly-ubiquitin (CST, 8081) and protein of interest were performed. A measure of 10% of the co-IP homogenate was used for examination of the input and loading control.

Proximity Ligation Assay

In situ protein-protein complexes and post-translational modifications (ubiquitination, neddylation) were measured using a proximity ligation assay (PLA, Duolink Sigma Aldrich).[67] OCT embedded

myocardial tissue sections of 5 µm were obtained and fixed with 4% PFA. Rabbit PLUS and mouse MINUS Duolink probes (Sigma Aldrich) were utilized to detect complexes using the antibodies targeting HIF1α (Novus Biologicals, NB100-479), HIF2α (Novus Biologicals, NB100-122), MDM2 (Novus Biologicals, NB100-2736), VHL (Invitrogen, PA5-27322), Ubiquitin mouse (SCBT, sc8017), Ubiquitin rabbit (CST, 3933) and NEDD8 (CST, 2754). Uniform antibody concentrations were maintained across different PLA experiments. Control experiments were performed using single antibody combinations and PLA probes alone to confirm that there was minimal non-selective detection at the antibody concentrations utilized **(Figure S3A-B, S3N, S4A-B, S4E, S4H**). Images were obtained at 20x and 40x magnification using fluorescence microscopy (Zeiss Axioplan). Images were analyzed in ImageJ to calculate the protein-protein complexes and nuclei per field.

In vivo Pharmacological Studies

For administration of the proteasome inhibitor Bortezomib (MCE, HY-10227, 0.1 mg/kg, daily injections from P1 to P6), pan PHD Inhibitor Molidustat (MCE, BAY 85-3934, 10 mg/kg, daily injections from P1 to P6) and MDM2 degrader MD-224(Adooq, A18712, 10 mg/kg, daily injections from P1 to P6 or P1 to P24), the drugs were dissolved in 1% DMSO and administered via subcutaneous route. Tamoxifen (50 mg/kg, injections at P1 and P4) was dissolved in warm sesame oil and administered via subcutaneous route. For information on frequency and interval, please refer to figure legends.

Echocardiography

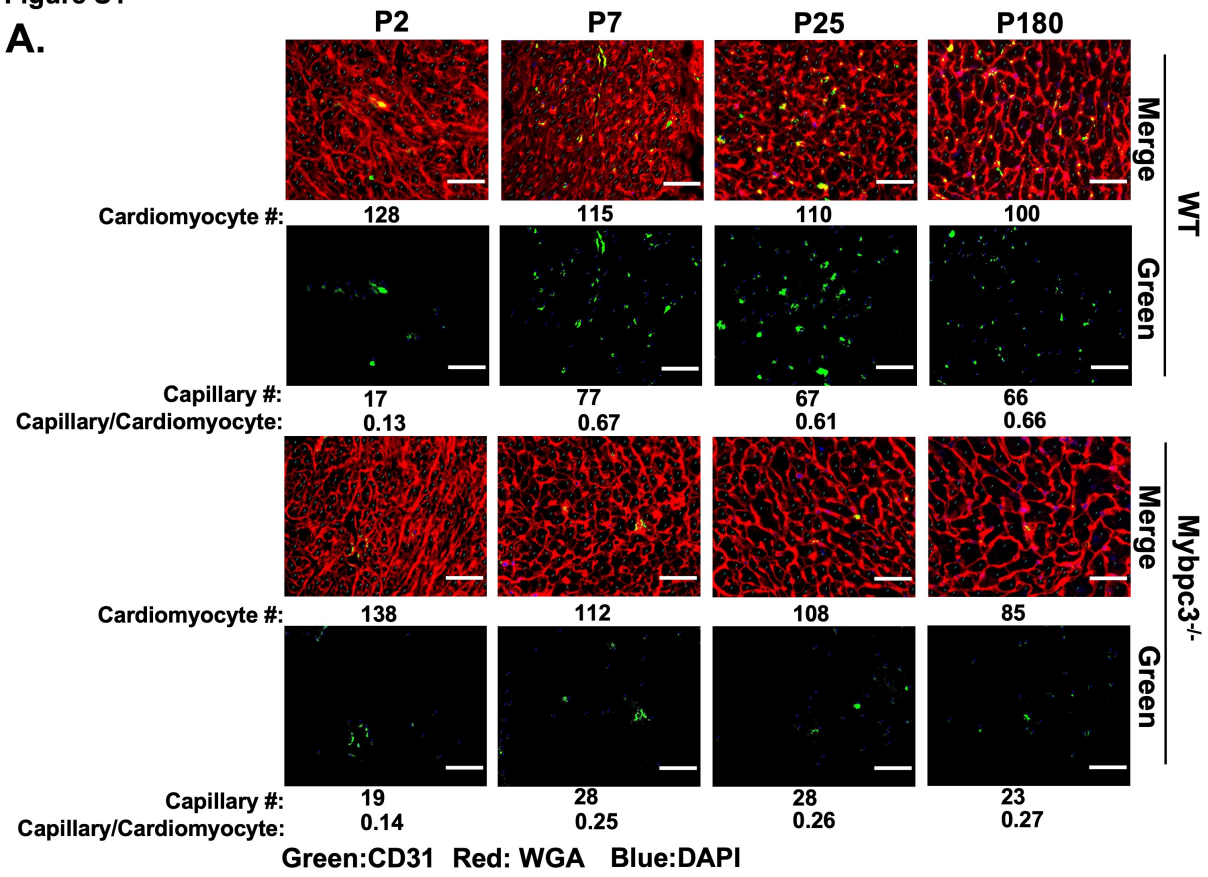
Murine cardiac structure and function were assessed by transthoracic echocardiography using a Vevo 3100 (VisualSonics Inc) without anesthesia. LV M-mode images were acquired to measure interventricular septal thickness at end diastole (IVSd, mm), LV posterior wall thickness at end diastole (LVPWd, mm), LV internal diameter at end diastole (LVIDd, mm) and LV internal diameter at end systole (LVIDs, mm). LV fractional shortening (FS, %) was calculated using the following

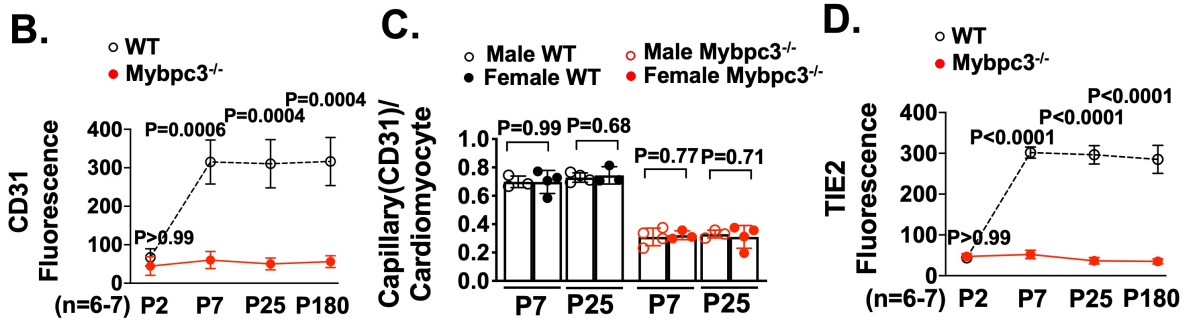
formula: [(LVIDd–LVIDs)/ LVIDd] x100. Quantification was performed in a blinded fashion using Vevo Lab or ImageJ/Fiji software.

Cardiomyocyte Size

OCT embedded myocardial tissue sections of 5 μm were obtained and fixed with 4% PFA. Following PBS washes, slides were incubated with Oregon Green 488–conjugated WGA (Thermo Fisher Scientific, W6748) diluted 1:200 in PBS. After PBS rinses, slides were mounted with ProlongGold Antifade with DAPI and LV tissue imaged at 40x magnification. Cardiomyocyte cross sectional area was then calculated using regions of interest (ROI) function in ImageJ software and the average cardiomyocyte cross-sectional area for each region was tabulated.

Figure S1





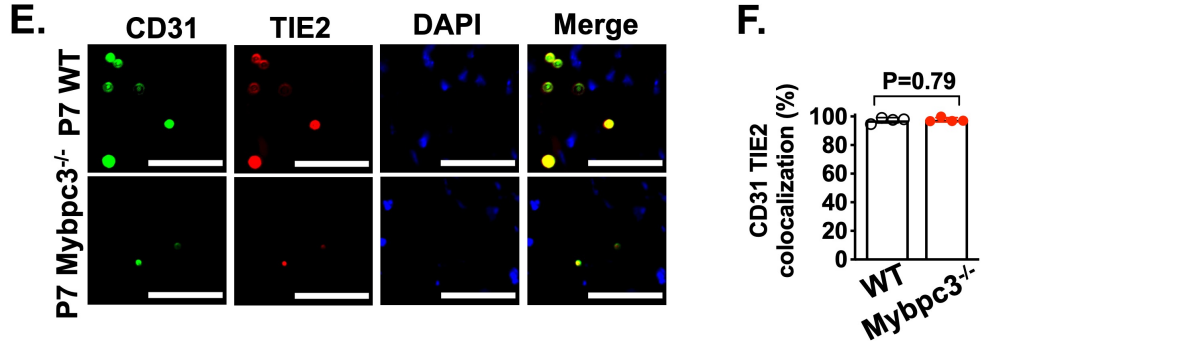


Figure S1 (A) Representative immunohistochemistry images for endothelial cell marker CD31 (green) co-stained with WGA (red) in LV tissue from WT and Mybpc3$^{-/-}$ mice at post-natal day 2 (P2), P7, P25 or P180. Nuclei are blue (DAPI). Cardiomyocytes (marked with white dot) were counted from WGA images and capillaries (marked with blue dot) were counted from CD31 images. Scale bars=40 µm. **(B)** Total fluorescence of immunohistochemistry images for CD31 in LV tissue from WT (n=6) and Mybpc3$^{-/-}$ (n=7) mice at P2, P7, P25 or P180. Average fluorescence of 3 non-overlapping LV images/sample. **(C)** Capillary to cardiomyocyte ratios in LV tissue from WT (male n=3-4, female n=3-4) and Mybpc3$^{-/-}$ (male n=3-4, female n=3-4) at P7 or P25. Minimum 120 cardiomyocytes/sample. **(D)** Total fluorescence of immunohistochemistry images for TIE2 in LV tissue from WT (n=6) and Mybpc3$^{-/-}$ (n=6-7) mice at P2, P7, P25 or P180. Average fluorescence of 3 non-overlapping LV images/sample. **(E)** Representative immunohistochemistry images for CD31 (green) and TIE2 (red) in LV tissue from P7 WT and Mybpc3$^{-/-}$ mice. Nuclei are blue (DAPI). Scale bars=30 µm. **(F)** CD31-TIE2 colocalization in LV tissue from WT (n=4) and Mybpc3$^{-/-}$ (n=4) mice. Minimum 100 endothelial cells/samples. All results are shown as mean±SEM. Student's t-test was utilized for C and F. Two-way ANOVA with Tukey multiple comparison test was utilized for B and D.

Figure S2

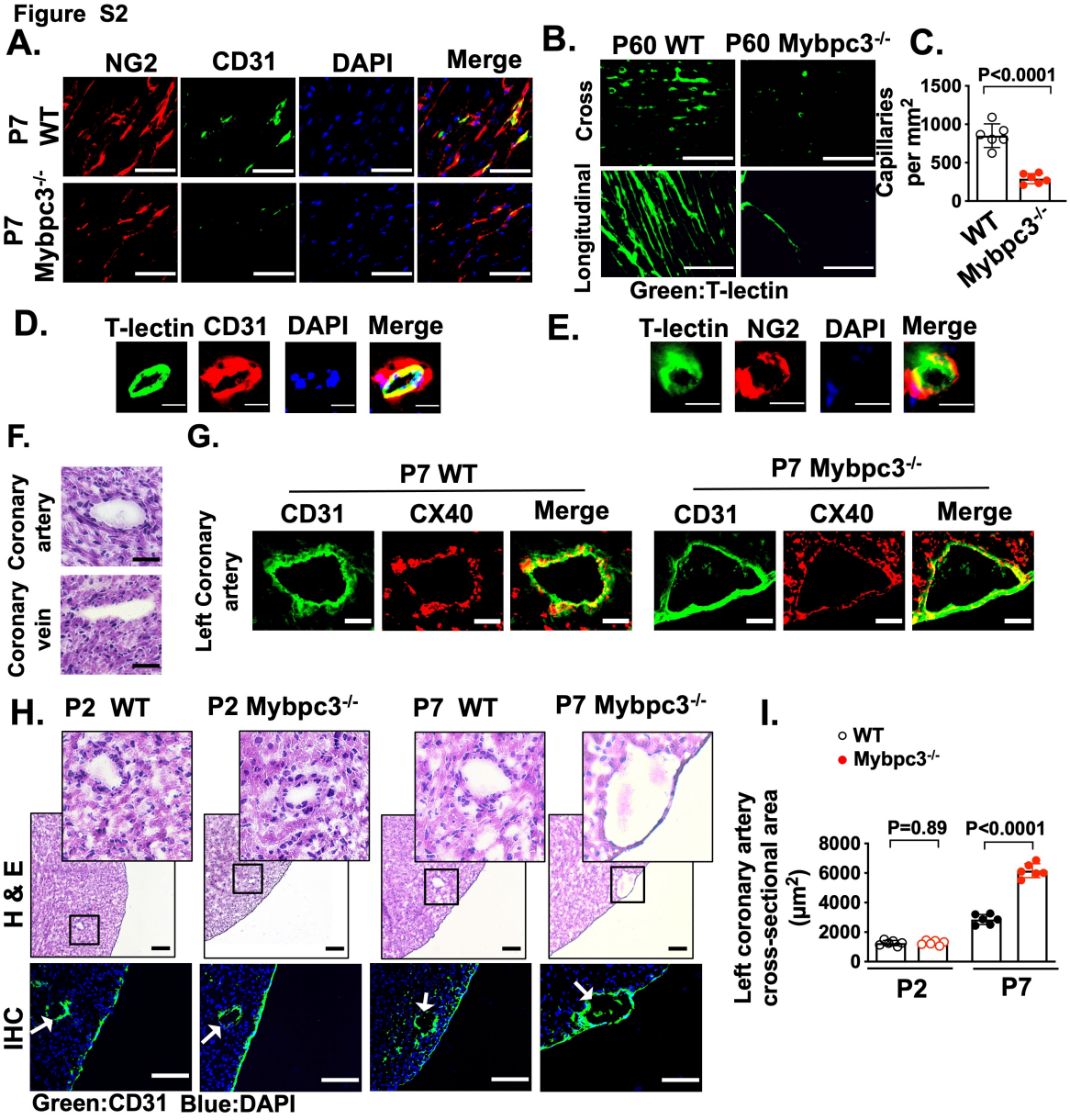


Figure S2 (A) Representative immunohistochemistry images for NG2 (red) and CD31 (green) in LV tissue from P7 WT and Mybpc3$^{-/-}$ mice. Nuclei are blue (DAPI). Scale bars=40 μm. **(B)** Representative fluorescence images for the intravascularly injected endothelial cell stain tomato lectin (T-Lectin) (green) in LV tissue cross-sections or longitudinal-sections from P60 WT and Mybpc3$^{-/-}$ mice. Scale bars=80 μm. **(C)** Capillaries per mm^2 in LV tissue from P7 WT (n=6) and Mybpc3$^{-/-}$ (n=6) mice. Three cross-sectional images per sample were analyzed. **(D)** Representative fluorescence images for the intravascularly injected endothelial cell stain tomato lectin (T-Lectin) (green) followed by immunohistochemistry for CD31 (red) in LV tissue from P7 WT mice. Scale bars=10 μm **(E)** Representative fluorescence images for the intravascularly injected endothelial cell stain tomato lectin (T-Lectin) (green) followed by immunohistochemistry for NG2 (red) in LV tissue from P7 WT mice. Scale bars=10 μm. **(F)** Representative H&E–stained LV tissue cross-sections showing coronary artery and vein from P7 WT mice. Scale bars=30 μm. **(G)** Representative immunohistochemistry images for CD31 (green) and CX40 (red) of left coronary artery (LCA) in LV tissue from P7 WT and Mybpc3$^{-/-}$ mice. Scale bars=30 μm. **(H)** [Top] Representative H&E–stained mid-ventricular LV tissue cross-sections with LCA magnified in WT and Mybpc3$^{-/-}$ mice at P2 or P7. **(H)** [Bottom] Representative immunohistochemistry images for CD31 (green) with arrow indicating the LCA in LV tissue from WT and Mybpc3$^{-/-}$ mice at P2 or P7. Scale bars=100 μm. **(I)** LCA cross-sectional area from LV tissue in WT (n=6) and Mybpc3$^{-/-}$ (n=6) mice at P2 or P7. Three independent mid-ventricular images/sample. **(J)** Representative B-mode echocardiographic images of the proximal LCA from P60 Mybpc3$^{-/-}$ mice with pulsed wave Doppler imaging (left) and without pulsed wave Doppler (right). Aorta (AO) outlined in red and LCA (arrow) outlined in white. LCA internal diameter noted by blue line. **(K)** Representative myocardial blood flow velocity tracing in LCA using pulsed wave Doppler. **(L)** Representative myocardial blood flow velocity tracing with diastolic specific velocity time integral (VTI) outlined. All results are shown as mean±SEM. Student's t-test was utilized for C and I.

Figure S3

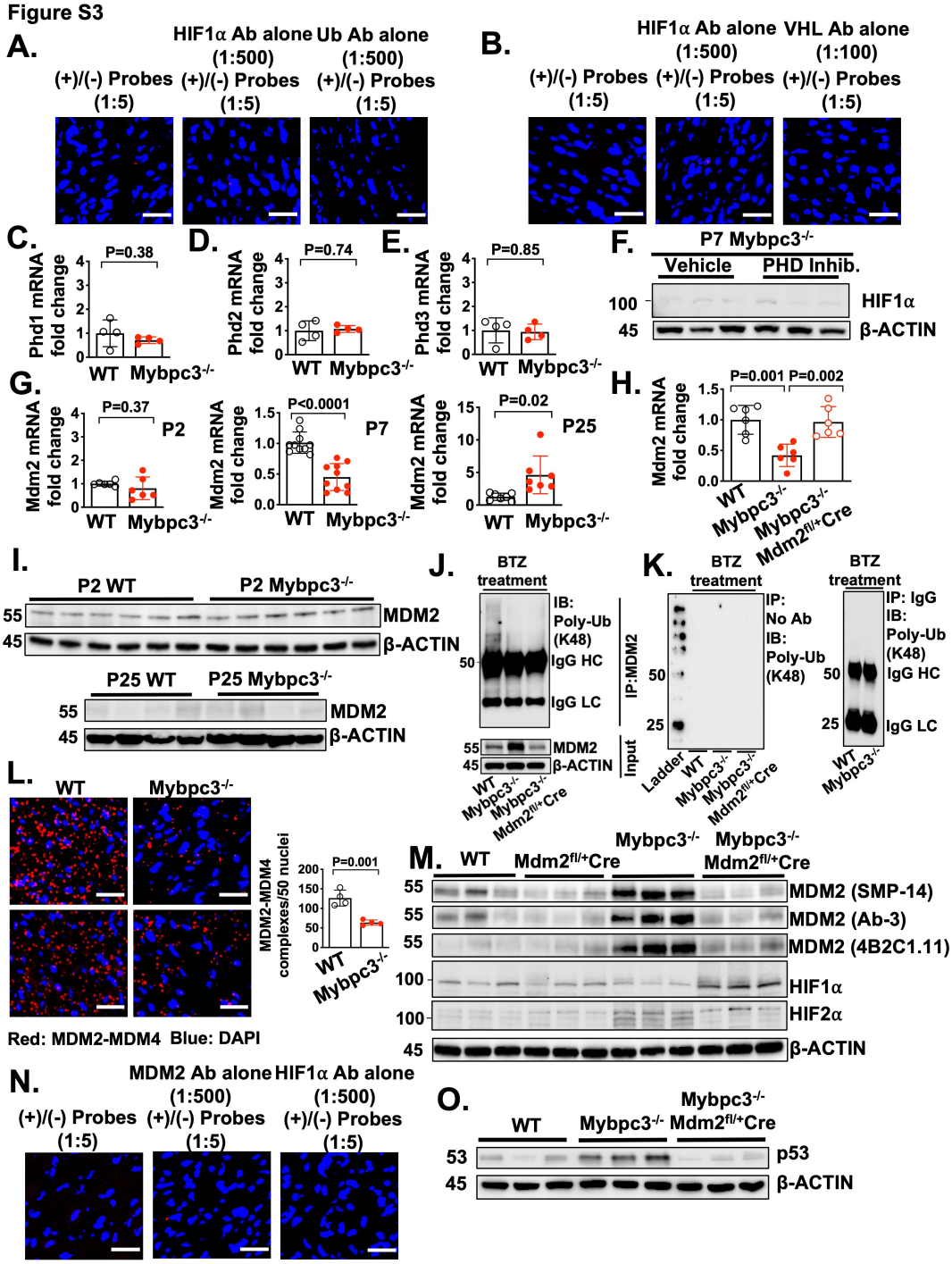


Figure S3 (A) Representative in situ proximity ligation assay (PLA) images for secondary antibody PLA probes only (+/-), HIF1α antibody and probes, and Ub antibody and probes in LV tissue from P7 WT mice. Non-specific complexes are red, and nuclei are blue (DAPI). Scale bars=25 µm. **(B)** Representative in situ PLA images for secondary antibody PLA probes only (+/-), HIF1α antibody and probes, and Von Hippel Lindau (VHL) antibody and probes in LV tissue from P7 WT mice. Non-specific complexes are red, and nuclei are blue (DAPI). Scale bars=25 µm. **(C-E)** Gene expression for Prolyl domain hydroxylase 1 (*Phd1*) **(C),** *Phd2* **(D)** and *Phd3* **(E)** in LV tissue RNA from P7 WT (n=4) and Mybpc3$^{-/-}$ (n=4) mice. The genes of interest were normalized to *Rpl32* and fold changes are relative to WT. **(F)** Immunoblots for HIF1α and β-actin in LV tissue from P7 Mybpc3$^{-/-}$ vehicle and Mybpc3$^{-/-}$ injected with PHD inhibitor (Molidustat, 10 mg/kg) from P1 to P6. **(G)** *Mdm2* gene expression in LV tissue RNA from WT (n=6-10) and Mybpc3$^{-/-}$ (n=6-10) mice at P2, P7 and P25. Mdm2 gene expression was normalized to *Rpl32* and fold change relative to WT. **(H)** *Mdm2* gene expression in LV tissue RNA from P7 WT (n=6), Mybpc3$^{-/-}$ (n=6) and Mybpc3$^{-/-}$Mdm2$^{fl/+}$/Myh6:Cre (n=6) mice. *Mdm2* gene expression was normalized to *Rpl32* and fold change relative to WT. **(I)** Immunoblot for murine double minute 2 (MDM2) LV tissue from WT (n=4-6) and Mybpc3$^{-/-}$ (n=4-6) mice at P2 and P25. **(J)** Immunoprecipitation (IP) for MDM2 was performed on LV tissue lysates from BTZ injected P7 WT, Mybpc3$^{-/-}$ and Mybpc3$^{-/-}$Mdm2$^{fl/+}$/Myh6:Cre mice and then immunoblots were performed for K48-linked ubiquitin (Poly-Ub). The input LV tissue lysates also underwent immunoblotting for MDM2 and β-actin. **(K) Left Blot**: Bead only precipitation without antibody was performed on LV tissue lysates from BTZ injected P7 WT, Mybpc3$^{-/-}$ and Mybpc3$^{-/-}$Mdm2$^{fl/+}$/Myh6:Cre mice and then immunoblots were performed for K48-linked ubiquitin (Poly-Ub). **Right Blot**: Immunoprecipitation (IP) for IgG was performed on LV tissue lysates from BTZ injected P7 WT and Mybpc3$^{-/-}$ mice and then immunoblots were performed for K48-linked ubiquitin (Poly-Ub). **(L)** Representative in situ PLA images and quantification for MDM2 and MDM4 complexes in LV tissue from P7 WT (n=4) and Mybpc3$^{-/-}$ (n=4) mice. MDM2-MDM4 complexes are shown in red and nuclei are blue (DAPI). Three non-overlapping LV images/sample. Scale bars=25 µm. **(M)** Immunoblots for murine double minute (MDM2) using three different MDM2 antibodies (clone SMP14, clone Ab-3, clone 4B2C1.11), HIF1α, HIF2α and β-actin in LV tissue from P7 WT, Mdm2$^{fl/+}$/Myh6:Cre, Mybpc3$^{-/-}$ and Mybpc3$^{-/-}$Mdm2$^{fl/+}$/Myh6:Cre mice. **(N)** Representative in situ PLA images for secondary antibody PLA probes only (+/-), MDM2 antibody and probes, and HIF1α antibody and probes in LV tissue from P7 WT mice. Non-specific complexes are red and nuclei are blue (DAPI). Scale bars=25 µm. **(O)** Immunoblot for p53 and β-actin in LV tissue from P7 WT, Mybpc3$^{-/-}$ and Mybpc3$^{-/-}$Mdm2$^{fl/+}$/Myh6:Cre mice. All results are shown as mean±SEM. Student's or Welch's t-test were utilized for C, D, E, G, and L. One-way ANOVA with Tukey multiple comparison test was utilized for H.

Figure S4

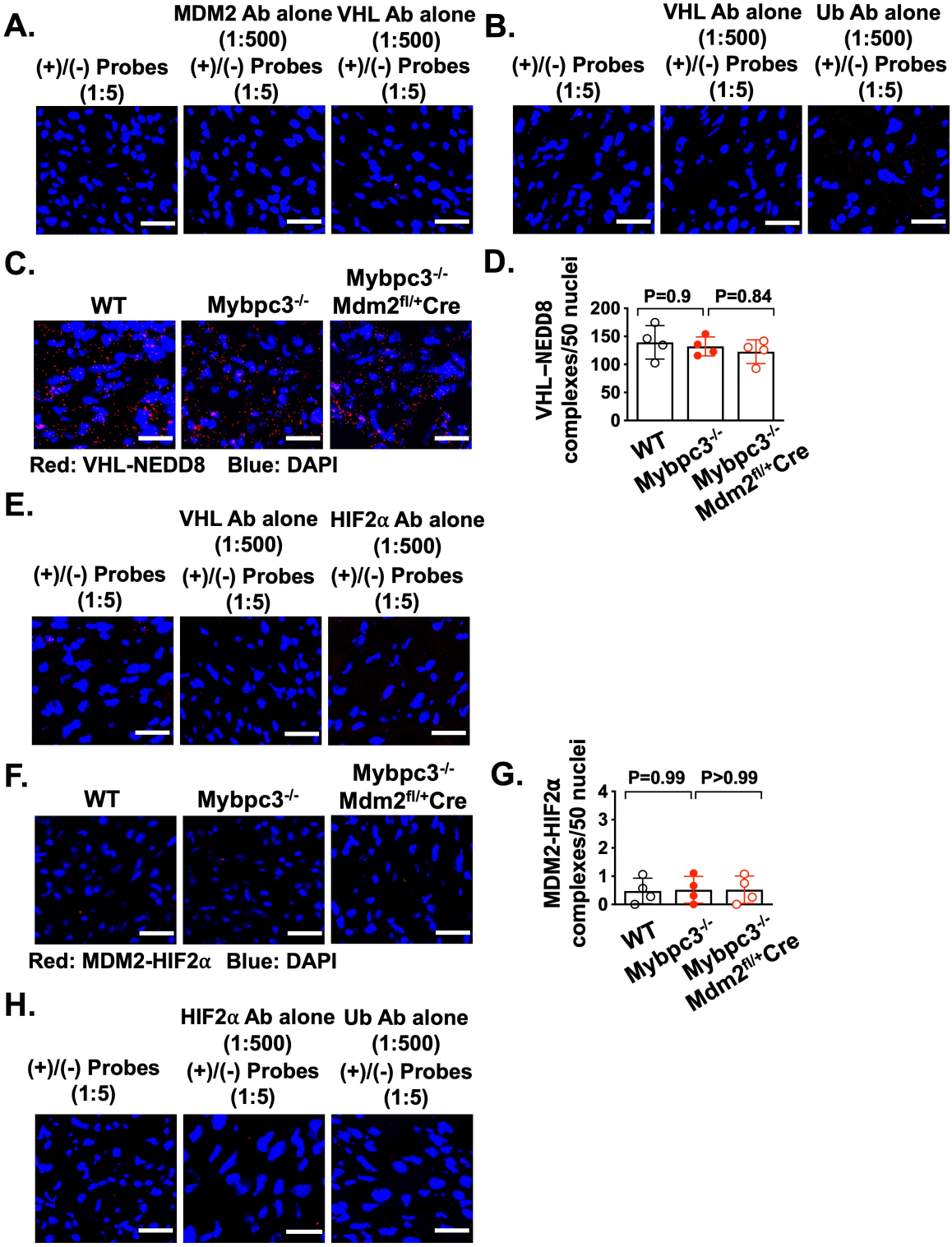


Figure S4 (A) Representative in situ proximity ligation assay (PLA) images for secondary antibody PLA probes only (+/-), MDM2 antibody and probes, and VHL antibody and probes in LV tissue from P7 WT mice. Non-specific complexes are red, and nuclei are blue (DAPI). Scale bars=25 μm. **(B)** Representative in situ PLA images for secondary antibody PLA probes only (+/-), VHL antibody and probes, and Ub antibody and probes in LV tissue from P7 WT mice. Non-specific complexes are red, and nuclei are blue (DAPI). Scale bars=25 μm. **(C+D)** Representative in situ PLA images and quantification for NEDD8 modified VHL in LV tissue from P7 WT (n=4), Mybpc3$^{-/-}$ (n=4) and Mybpc3$^{-/-}$Mdm2$^{fl/+}$/Myh6:Cre (n=4) mice. VHL-NEDD8 complexes are red, and nuclei are blue (DAPI). Scale bars=25 μm. Three non-overlapping LV images/sample. **(E)** Representative in situ PLA images for secondary antibody PLA probes only (+/-), VHL antibody and probes, and HIF2α antibody and probes in LV tissue from P7 WT mice. Non-specific complexes are red, and nuclei are blue (DAPI). Scale bars=25 μm. **(F+G)** Representative in situ PLA images and quantification for MDM2 and HIF2α protein complexes in LV tissue from P7 WT (n=4), Mybpc3$^{-/-}$ (n=4) and Mybpc3$^{-/-}$Mdm2$^{fl/+}$/Myh6:Cre (n=4) mice. MDM2-HIF2α protein complexes are red, and nuclei are blue (DAPI). Scale bars=25 μm. Three non-overlapping LV images/sample. **(H)** Representative in situ PLA images for secondary antibody PLA probes only (+/-), HIF2α antibody and probes, and Ub antibody and probes in LV tissue from P7 WT mice. Non-specific complexes are red, and nuclei are blue (DAPI). Scale bars=25 μm. All results are shown as mean±SEM. One-way ANOVA with Tukey multiple comparison test was utilized for D and G.

Figure S5

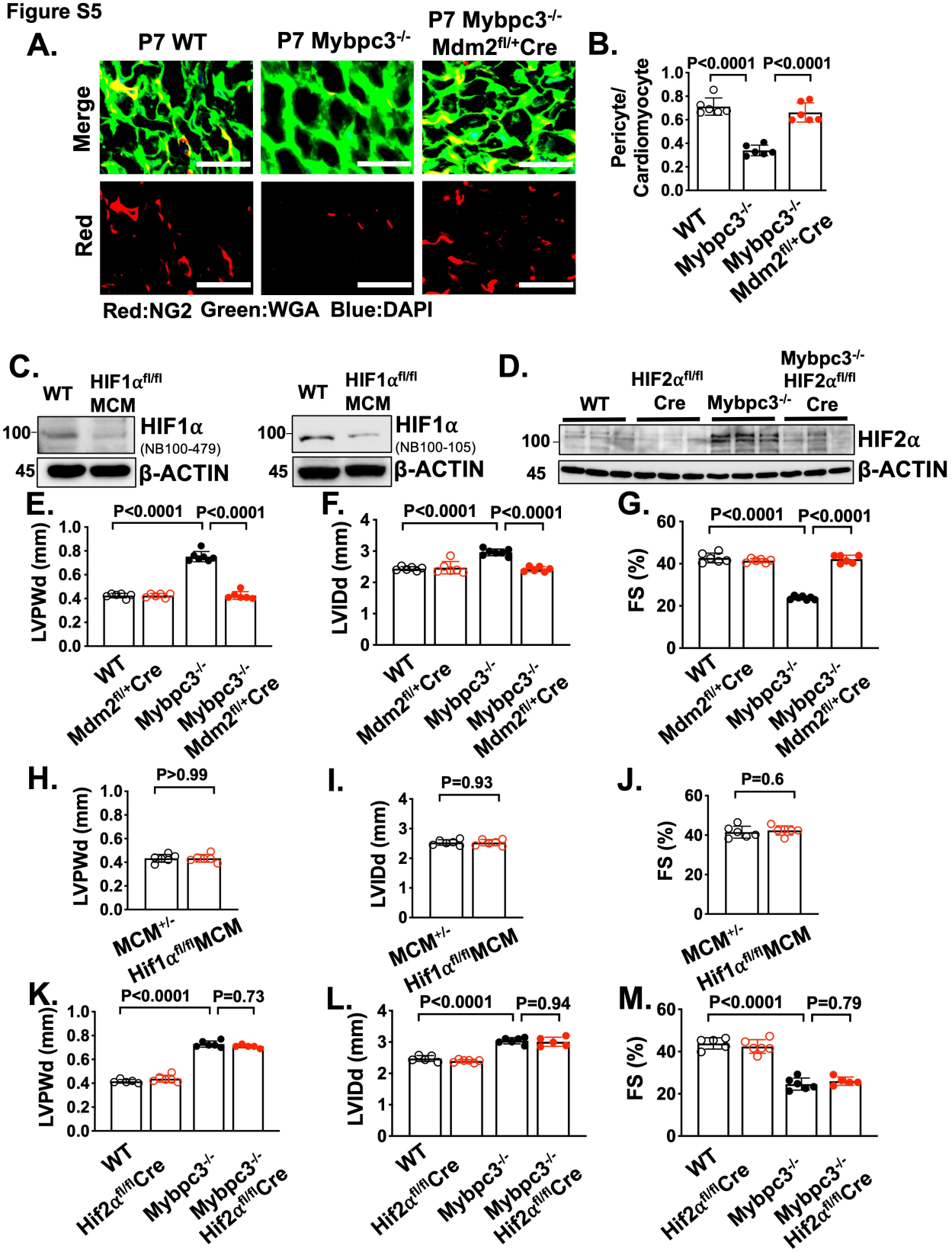


Figure S5 (A) Representative immunohistochemistry images for pericyte marker NG2 (red) and counterstained for WGA (green) in LV tissue from P7 WT, Mybpc3$^{-/-}$ and Mybpc3$^{-/-}$Mdm2$^{fl/+}$/Myh6:Cre mice. Nuclei are blue (DAPI). Scale bars=25 μm. **(B)** Pericyte to cardiomyocyte ratios in LV tissue from P7 WT (n=6), Mybpc3$^{-/-}$ (n=6) and Mybpc3$^{-/-}$Mdm2$^{fl/+}$/Myh6:Cre (n=6) mice. Minimum 200 cardiomyocytes/sample. **(C)** Immunoblots for HIF1α and β-actin in LV tissue from WT and Hif1α$^{fl/fl}$/Myh6:MerCreMer mice injected with tamoxifen at P1 and P4. Left panel - NB100-479 HIF1α antibody, Right panel – NB100-105 HIF1α antibody. **(D)** Immunoblots for HIF2α and β-actin in LV tissue from P7 WT, Hif2α$^{fl/fl}$/Myh6:Cre, Mybpc3$^{-/-}$, and Mybpc3$^{-/-}$Hif2α$^{fl/fl}$/Myh6:Cre mice. **(E-G)** Echocardiography assessment of **(E)** LV posterior wall thickness at end diastole (LVPWd), **(F)** LV internal diameter at end diastole (LVIDd) and **(G)** LV fractional shortening (FS) in P7 WT (n=6), Mdm2$^{fl/+}$/Myh6:Cre (n=6), Mybpc3$^{-/-}$ (n=7), and Mybpc3$^{-/-}$Mdm2$^{fl/+}$/Myh6:Cre (n=6) mice. **(H-J)** Echocardiography assessment of **(H)** LVPWd, **(I)** LVIDd and **(J)** FS in P7 MCM (n=6) and Hif1α$^{fl/fl}$/Myh6:MerCreMer (n=6) mice injected with tamoxifen at P1 and P4. **(K-M)** Echocardiography assessment of **(K)** LVPWd, **(L)** LVIDd and **(M)** FS in P7 WT (n=5), Hif2α$^{fl/fl}$/Myh6:Cre (n=6), Mybpc3$^{-/-}$ (n=6), and Mybpc3$^{-/-}$Hif2α$^{fl/fl}$/Myh6:Cre (n=5) mice. All results are shown as mean±SEM. One-way ANOVA with Tukey multiple comparison test was utilized for B. Student's t-test was utilized for H-J. Two-way ANOVA with Tukey multiple comparison test was utilized for E-G and K-M.

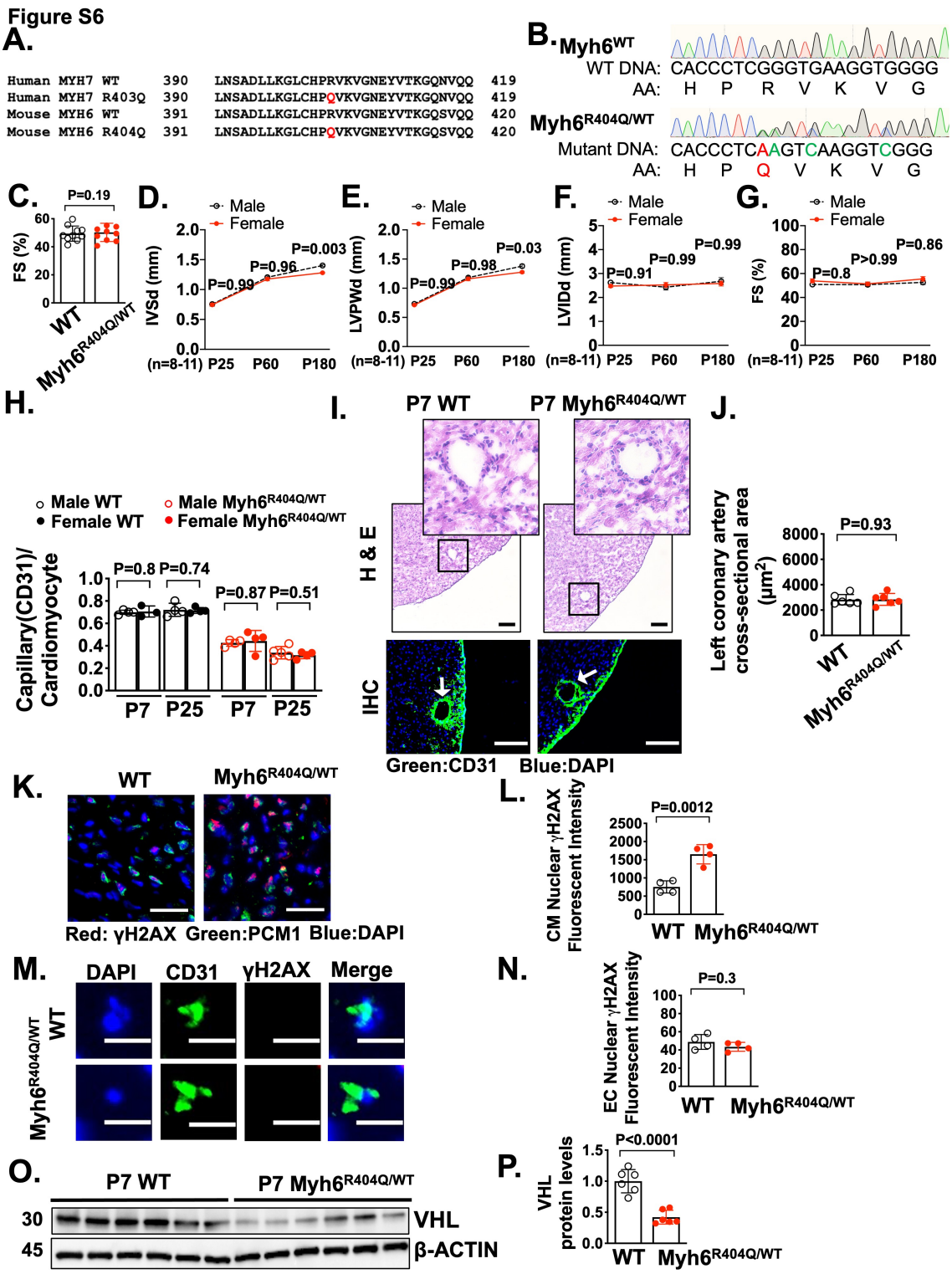
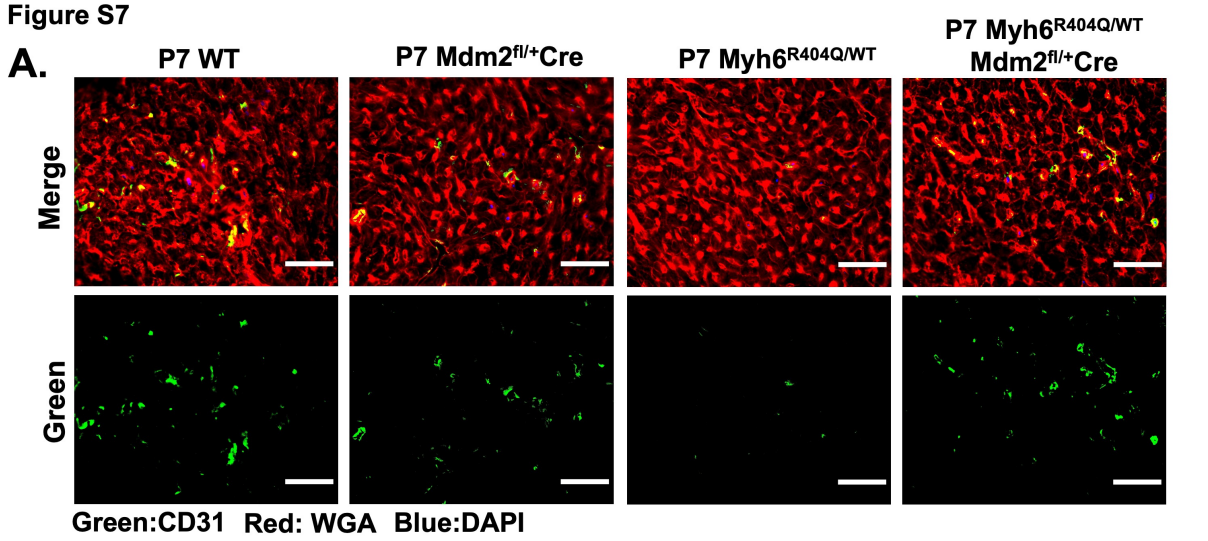
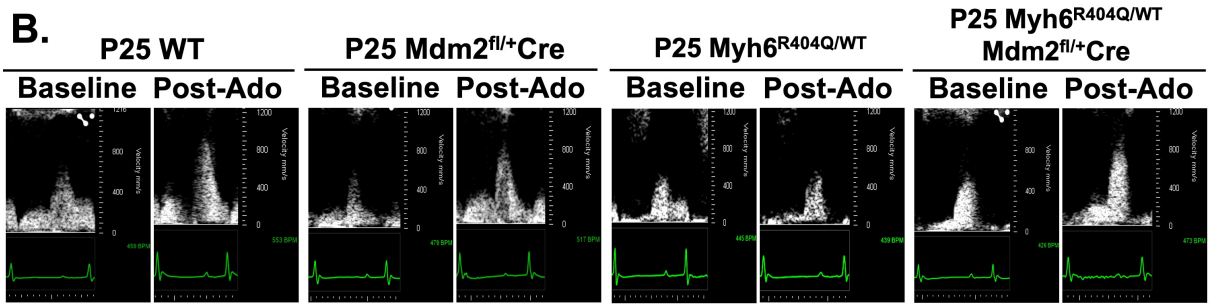
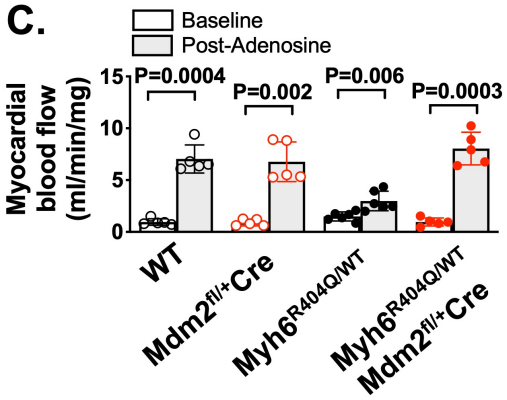


Figure S6 (A) Amino acid comparison between human MYH7 WT and R403Q mutation and the mouse MYH6 WT and R404Q mutation. **(B)** Chromatogram of DNA sequencing from WT and Myh6$^{R404Q/WT}$ mice. The mutated base pairs in red create the R404Q amino acid change and the base pairs in green are silent DNA changes which facilitate genotyping and prevent CRISPR/Cas9 mediated cutting of the donor DNA. **(C)** Fractional shortening (FS) in P60 WT (n=10) and Myh6$^{R404Q/WT}$ (n=9) mice. **(D-G)** Echocardiography assessment of **(D)** interventricular septal thickness at end diastole (IVSd), **(E)** left ventricular [LV] posterior wall thickness at end diastole (LVPWd), **(F)** LV internal diameter at end diastole (LVIDd), and **(G)** FS in male (n=8-11) and female (n=8-11) Myh6$^{R404Q/WT}$ mice at P25, P60 or P180. **(H)** Capillary to cardiomyocyte ratios in LV tissue from WT (male n=4, female n=3-4) and Myh6$^{R404Q/WT}$ (male n=4-5, female n=4) mice at P7 or P25. Minimum 100 cardiomyocytes/sample. **(I)** Representative H&E–stained heart cross-sections of P7 WT and Myh6$^{R404Q/WT}$ mice showing left coronary artery (LCA) [top]. Representative immunohistochemistry images for CD31 (green) with LCA (arrow) in LV tissue from P7 WT and Myh6$^{R404Q/WT}$ mice [bottom]. Scale bars=100 μm. **(J)** LCA cross-sectional area in LV tissue from P7 WT and Myh6$^{R404Q/WT}$ mice (n=6/group). Three independent mid-ventricular images/sample. **(K)** Representative immunohistochemistry images for the DNA damage marker γH2AX (red) and cardiomyocyte (CM) nuclei marker pericentriolar material 1 (PCM1)(green) in LV tissue from P7 WT and Myh6$^{R404Q/WT}$ mice. Nuclei are blue (DAPI). Scale bars=25 μm. **(L)** CM nuclear γH2AX fluorescence in LV tissue from P7 WT (n=4) and Myh6$^{R404Q/WT}$ (n=4) mice. Minimum 100 CM nuclei/sample. **(M)** Representative immunohistochemistry images for γH2AX (red) and endothelial cell (EC) marker CD31 (green) in LV tissue from P7 WT and Mybpc3$^{-/-}$ mice. Nuclei are blue (DAPI). Scale bars=10 μm. **(N)** EC nuclear γH2AX fluorescence in LV tissue from P7 WT (n=4) and Mybpc3$^{-/-}$ (n=4) mice. Minimum 50 EC nuclei/sample. **(O+P)** Immunoblots and quantification for VHL in LV tissue lysates from P7 WT (n=6) and Myh6$^{R404Q/WT}$ (n=6) mice normalized to β-actin and relative to WT. Student's t-test was utilized for C, H, J, L, N and P. Two-way ANOVA with Tukey multiple comparison test was utilized for D, E, F, and G.

Figure S7







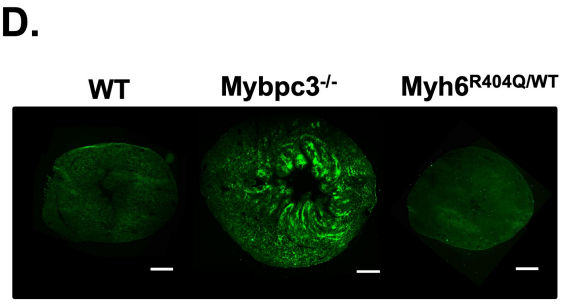


Figure S7 (A) Representative immunohistochemistry images for CD31 (green) co-stained with WGA (red) in LV tissue from P7 WT, Mdm2$^{fl/+}$Cre, Myh6$^{R404Q/WT}$ and Myh6$^{R404Q/WT}$Mdm2$^{fl/+}$Cre mice. Nuclei are blue (DAPI). Scale bars=40 μm. **(B)** Representative myocardial blood flow velocity tracings from pulsed wave Doppler echocardiography of P25 WT, Mdm2$^{fl/+}$Cre, Myh6$^{R404Q/WT}$ and Myh6$^{R404Q/WT}$Mdm2$^{fl/+}$Cre mice. Myocardial blood flow before (baseline) and after injection with adenosine (post-ado) to induce maximal hyperemia. **(C)** Myocardial blood flow in P25 WT (n=5), Mdm2$^{fl/+}$Cre (n=5), Myh6$^{R404Q/WT}$ (n=6) and Myh6$^{R404Q/WT}$Mdm2$^{fl/+}$Cre (n=5) mice. Myocardial blood flow before adenosine (white bars) and post-adenosine (light grey bars) was calculated and normalized to heart weight. All results are shown as mean±SEM. **(D)** Representative immunohistochemistry images of heart cross sections from P7 WT , Mybpc3$^{-/-}$ and Myh6$^{R404Q/WT}$ mice injected with pimonidazole (hypoxyprobe, green). Scale bars=0.5 mm. Student's or Welch's t-test were utilized for C.

Figure S8

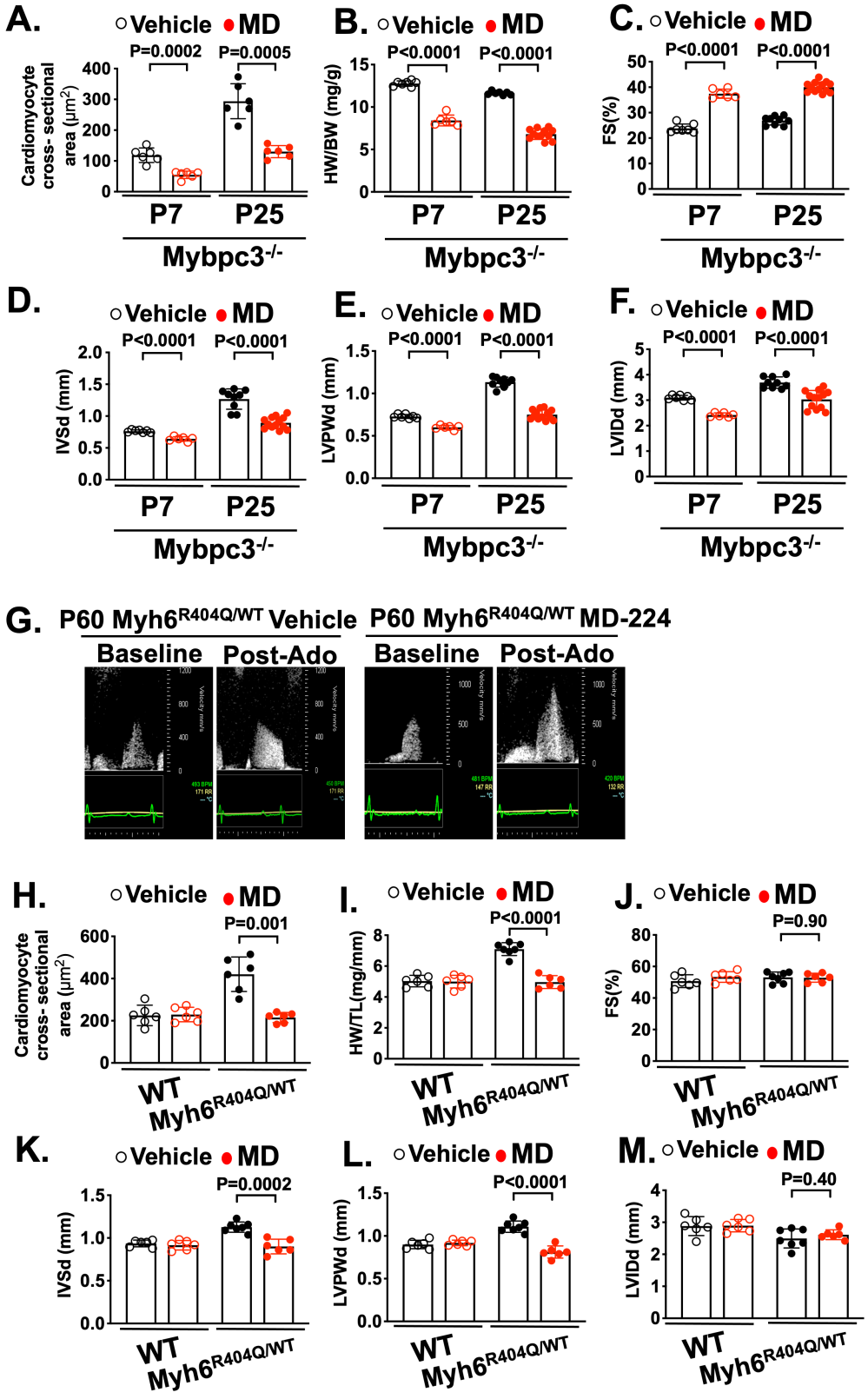


Figure S8 (A) Cardiomyocyte cross-sectional areas from WGA stained LV tissue from P7 and P25 Mybpc3$^{-/-}$ (n=6) mice injected with vehicle or MD-224 (MD). Minimum 50 cardiomyocytes/sample. **(B)** Heart weight to body weight ratio (HW/BW) from P7 and P25 Mybpc3$^{-/-}$ (n=6-12) mice injected with vehicle or MD-224. Echocardiography assessment of **(C)** fractional shortening (FS), **(D)** interventricular septal thickness at end diastole (IVSd), **(E)** left ventricular [LV] posterior wall thickness at end diastole (LVPWd), and **(F)** LV internal diameter at end diastole (LVIDd) in P7 and P25 Mybpc3$^{-/-}$ (n=6-13) injected with vehicle or MD-224. **(G)** Representative myocardial blood flow velocity tracings from pulsed wave Doppler echocardiography of P60 Myh6$^{R404Q/WT}$ mice injected with vehicle or MDM2 PROTAC (MD-224) from P1 to P24. Myocardial blood flow before (baseline) and after retro-orbital injection with adenosine (post-ado) to induce maximal hyperemia. **(H)** Cardiomyocyte cross-sectional areas from WGA stained LV tissue from P60 WT and Myh6$^{R404Q/WT}$ (n=6) mice injected with vehicle or MD-224. Minimum 50 cardiomyocytes/sample. **(I)** Heart weight to tibial length ratio (HW/TL) from P60 WT (n=6) and Myh6$^{R404Q/WT}$ (n=6-7) mice injected with vehicle or MD-224. Echocardiography assessment of **(J)** fractional shortening (FS), **(K)** interventricular septal thickness at end diastole (IVSd), **(L)** left ventricular [LV] posterior wall thickness at end diastole (LVPWd), and **(M)** LV internal diameter at end diastole (LVIDd) in P60 WT (n=6) and Myh6$^{R404Q/WT}$ (n=6-7) injected with vehicle or MD-224. Student's or Welch's t-test were utilized for A-F and H-M.

Figure S9

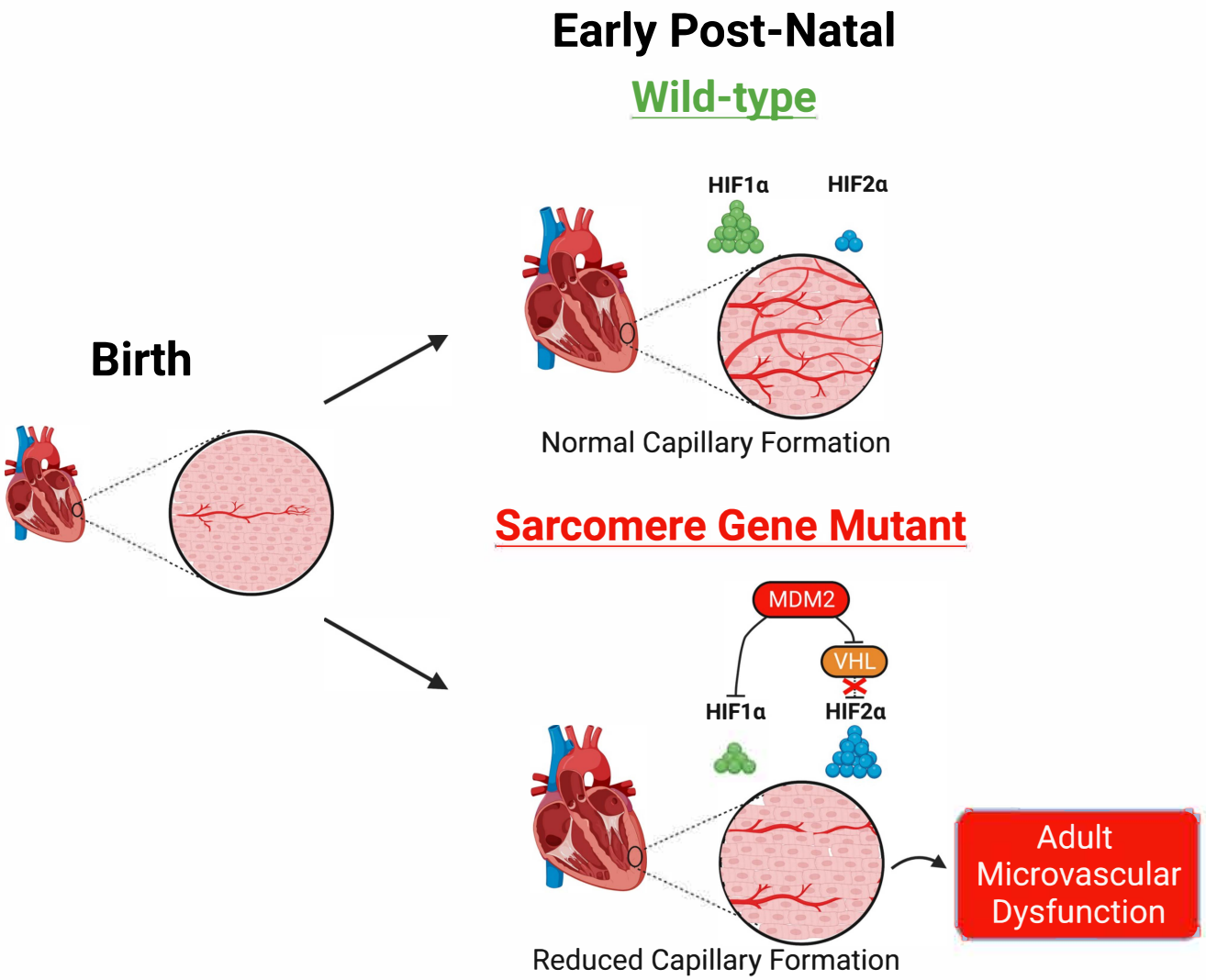

Early Post-Natal
Wild-type
HIF1α
HIF2α
Birth
Normal Capillary Formation
Sarcomere Gene Mutant
MDM2
VHL
HIF1α
HIF2α
Adult Microvascular Dysfunction
Reduced Capillary Formation

Table S1

Myh6 sgRNA (chr14:54,960,460-54,960,479 Mouse Dec. 2011 GRCm38/mm10 Assembly)
5'-TGTCACCCTCGGGTGAAGGT-3'
Myh6 R404Q mutant DNA template (mutation underlined and silent substitutions bolded)
AATCTGCCTACCTTATGGGGCTGAACTCAGCTGACCTGCTCAAGGGCCTGTGTCACCCT C**A**A**A**GT**C**AAGGT**C**GGGAACGAGTATGTCACCAAGGGGCAGAGTGTACAGCAAGTGTACT ATTCCATCGGGGCACTGG
Myh6 WT DNA template (silent substitutions bolded)
AATCTGCCTACCTTATGGGGCTGAACTCAGCTGACCTGCTCAAGGGCCTGTGTCACCCT CG**A**GT**C**AA**A**GTGGGGAACGAGTATGTCACCAAGGGGCAGAGTGTACAGCAAGTGTACT ATTCCATCGGGGCACTGG

Table S2

Murine Gene	Forward Primer	Reverse Primer
Rpl32	CACCAGTCAGACCGATATGTGAAAA	TGTTGTCAATGCCTCTGGGTTT
Hif1α	GCAACGTGGAAGGTGCTT	AGGAATTTCAATATTTGACGGATG
Vhl	CCCAGGTCATCTTCTGCAAT	TTGGCAAAAATAGGCTGTCC
Hif2α	AACGGCTCTGGTTTTGGGAA	GTTGACAGTCCGGCCTCT
Vegfa	CGTCAGAGAGCAACATCACC	TCTGTCTTTCTTTGGTCTGCA
Vegfb	CCAGCCACCAGAAGAAAGTG	TTGTTTGACCACATTGCCCA
Vegfc	GCGAGGTCAAGGCTTTTGAA	CCCCTGTCCTGGTATTGAGG
Angpt1	CAGATGTTGAGACCCAGGTAC	TCTTCTTTGTGTTTTCCCTCCA
Angpt2	GCCCAAGTACTAAACCAGACG	GCTTGTTTATTTCACTGGTCTGA
Pgf	AGAAGTGGAAGTGGTGCCTT	ACTCAGAAGGACACAGGACG
Pdgfb	TCCGTAGATGAAGATGGGGC	TCCGAGAGATCTGGAACACC
Phd1	CATCAATGGGCGCACCA	GATTGTCAACATGCCTCACGTAC
Phd2	TAAACGGCCGAACGAAAGC	GCTTGCAGAAAGTATCCACTGT
Phd3	CTATGTCAAGGAGCGGTCCAA	GTCCACATGGCGAACATAACC
Mdm2	GGAGATCCATTAGTGAGACAGAAGA	AGACCCAGGCTCGGATCA