Supplemental Materials

Supplemental Methods

Generation of SM22-Cre;Dicer$^{fl/fl}$ Embryos

In initial experiments, heterozygous SM22αCre/Dicer$^{fl/wt}$ male mice were either bred with Cre negative Dicer$^{fl/fl}$ female mice or heterozygous SM22-Cre/Dicer$^{fl/wt}$ female mice. In subsequent timed breeding experiments, only the former breeding strategy was used. Females were checked daily for the presence of vaginal plug, which indicated E0.5. SM22αCre negative Dicer$^{fl/wt}$ embryos (D$^{fl/wt}$) were used as controls for SM22αCre/Dicer$^{fl/fl}$ embryos (Dicer KO). Genotyping was performed using PCR as reported previously [1].

Cell culture

At the age of 3 weeks, male MHCCreER^{T2}/Dicer$^{fl/fl}$ mice were treated with intraperitoneal injections of tamoxifen (50g/kg/day) for 5 consecutive days. Excision of the floxed Dicer allele was confirmed by genotyping and aortic smooth muscle cells were then isolated using a standard enzymatic digestion technique. Control cells were isolated simultaneously from age matched, tamoxifen treated, Cre negative Dicer$^{fl/fl}$ mice. Breifly, aortas were isolated and incubated for 30 min at 37^{o}C in serum free DMEM cell culture media containing 1 mg/ml collagenase type 2 (Worthington Biochemical Corporation). The adventitia was then pulled of using forceps and the aorta was incubated for 4h at 37^{o}C in serum free DMEM cell culture media containing 2 mg/ml collagenase type 2 (Worthington Biochemical Corporation) and 0.2mg/ml elastase (Sigma).

qRT-PCR

Quantitative real time PCR for Dicer and selected miRNAs was performed as reported previously [1, 2]. The qPCR primer sequences used for *Acta2, Cnn1, Myh11, Myocd, KLF4 and KLF5* have been published previously [3-5]. Commercially available primer sequences for selected miRNAs were used.

Adenoviral gene transfer

For adenoviral gene transfer of myocardin SMC were incubated overnight in DMEM / 10%FCS with Ad.MYOCD or Ad.LacZ at moi 100 [6]. After removal of the virus, SMCs were cultured for 36h in DMEM / 10%FCS.

Western blotting

The umbilical and vitelline artery and vein from each embryo was dissected free from surrounding tissue and snap frozen in liquid nitrogen. Standard western blot analysis was conducted using Dicer (Santa Cruz Biotechnology Inc.), HSP90 (BD Biosciences), α-actin (Sigma), SM-MHC (Biomedical Technologies Inc.), Calponin, SM22, KLF4, KLF5 (Abcam) antibodies.

[^{3}H]Thymidine incorporation

Control and Dicer KO SMC were starved for 36h, transfected for 15h with negative control or miR-145 mimic and then stimulated for 36h with 10% FCS with 1μCi [^{3}H]Thymidine (PerkinElmer). After washing and standard TCA-precipitation, the incorporated [^{3}H]Thymidine was quantified by liquid scintillography.

Histological analysis

Embryos generated by timed breeding were isolated, fixed in 4% paraformaldehyde, paraffin embedded and sectioned. The sections were stained with hematoxylin and eosin (H&E) and analyzed by microscopy. Freshly dissected whole embryos and organs were photographed using a dissecting microscope.

Immunofluorescence

Following deparaffinization and rehydration, slides were incubated in Tris-EDTA buffer (10mM Tris, 1mM EDTA, 0.05% Tween 20, pH 9) at 97°C for 15 min then blocked using 10% goat serum and incubated over night with antibodies against Ki67 (Abcam) and smooth muscle alpha actin (Sigma). For F-actin and G-actin staining, isolated smooth muscle cells, passage 4-5, were fixed in 4% paraformaldehyde for 10 min, permeabilized for 5 min in 0.1% triton and incubated for 30 min at room temperature with rhodamine phalloidin (Molecular Probes) for F-actin and alexa fluor 488 conjugated DNAse1 (Invitrogen) for G-actin.

β-Galactosidase staining of SM22Cre;ROSA26 embryos

Heterozygous SM22αCre female mice (The Jackson Laboratory) were timed bred with a homozygous ROSA26 (The Jackson Laboratory) male mouse. At E15.5 the embryos were dissected from the yolk sacs and opened at the thorax and abdomen to allow penetration of the staining solutions. Whole staining of embryos were then performed as previously described [7].

Electron microscopy

Embryos (E16.5; n=5) were fixed at 4^{o}C in 0.1M phosphate buffered 2.5% glutaraldehyde, postfixed 60 minutes in phosphate buffered 1.0% osmium tetroxide, dehydrated in a graded series of ethanol to 100 % (3 times), transitioned to propylene oxide, infiltrated with EPON/Araldite resin, embedded in fresh resin and polymerized for two days at 70^{o}C. Thin sections of the dorsal aorta (70nm) were placed onto 100 mesh copper grids, stained with uranyl acetate/lead citrate and analyzed in a blind manner with a Hitachi 7650 transmission electron microscope equipped with a GATAN Erlangshen 11 mega pixel digital camera. Final images were processed in Adobe Photoshop.

Force measurement

Active and passive circumference-tension relationships were then generated as previously described[8]. Briefly, vessels were contracted twice using High-KCl HEPES buffer obtained by exchanging 80 mM NaCl for KCl before circumference-tension relationships were generated by increasing wire distance in a stepwise fashion. At each wire distance, preparations were allowed to equilibrate in Ca^{2+}-HEPES for 5 min, then stimulated with High-KCl buffer for 6 min (active force) and relaxed in nominally Ca^{2+}-free solution for 10 min (passive force). Calcium chelating agents such as EGTA was not used in the Ca^{2+}-free solution since this may negatively affect subsequent contractile responses. However, previous experience shows that smooth muscle myogenic tone in nominally Ca^{2+}-free solution is negligible.

Supplemental Figure Legends

Supplemental Fig. I (A) Crown-to-rump length and wet weight of E16.5 embryos. (B) qPCR analysis of Dicer in umbilical cords of E14.5 embryos.

Supplemental Fig. II Paraffin embedded, H&E stained section of the abdominal area of $D^{fl/wt}$ and SMC Dicer KO E16.5 embryos. Four 4X photos were acquired and assembled into one montage. Note intraperitoneal hemorrhage in the SMC-Dicer KO embryo indicated by arrows. Areas of hemorrhage within the liver are indicated by asterisks.

Supplemental Fig. III (A) Organs isolated from E16.5 $D^{fl/wt}$ and SMC-Dicer KO embryos. Bar indicates 5mm. (B) Paraffin embedded, H&E stained section of E16.5 hearts. Bar indicates 500mm . (C) LacZ staining of tissues from SM22-Cre positive and Cre negative embryos carrying the ROSA26 reporter gene (A). Bl; bladder, UA; umbilical artery, Ao; aorta, Eso; esophagus, Tra; trachea.

Supplemental Fig. IV TEM showing a cross-section of a $D^{fl/wt}$ E15.5 dorsal aorta (top) and a similar region from a SMC-Dicer KO embryo (bottom). Note disordered array of medial SMC in the Dicer KO aorta and their less spindled morphology. Scale bars are indicated at bottom. Magnification is 6,000x.

Supplemental Fig. V (A) Analysis of outer and inner diameter of the aortic media of E12.5-E16.5 embryos shown in figure 3A. n=4-6 *=$p<0.05$ (B) ERK1/2 and Akt phosphorylation in umbilical cords of E16.5 embryos. n=3-5 *$p<0.05$. (C) Vascular

geometry of H&E stained frozen sections of E16.5 umbilical arteries. n=3. (D) H&E stained cross section of the umbilical arteries from E16.5 embryos. m indicates the smooth muscle media. (E) qPCR analysis of myocardin and KLF4 in umbilical cords of E16.5 embryos.

Supplemental Fig. VI (A) Analysis of mRNA expression of *Myocd, KLF4* and *KLF5* in Dicer KO and Ctrl SMC following 96h transfection of Neg Ctrl, miR-145, miR-21 or miR-221. (B) Ctrl and Dicer KO SMC transduced with Ad-lacZ or Ad-myocardin expressing adenoviral vectors for 48h. miR-145 and SMC specific genes (*Acta-2*: SM-a-actin, *Cnn1*: calponin, and *Myh11*: myosin heavy chain) were analyzed by qPCR. (C) Proliferation of Dicer KO and control SMC stimulated with 10% fetal calf serum (FCS) was analyzed using 3H-Thyminine incorporation. Cells were either transfected with Neg Ctrl or miR-145 mimic prior to FCS stimulation. n=3-5, *$p<0.05$

Supplemental Fig. VII Actin filaments (F-actin) and monomeric actin (G-actin) were visualized in Dicer KO (KO) and Ctrl SMC using rhodamine phalloidine (red) and Alexa 488 DNase1 (green), respectively. Upper four panels show representative images of Ctrl and Dicer KO cells using a 10X objective. Lower two panels show representative cells using a 63X objective.

References

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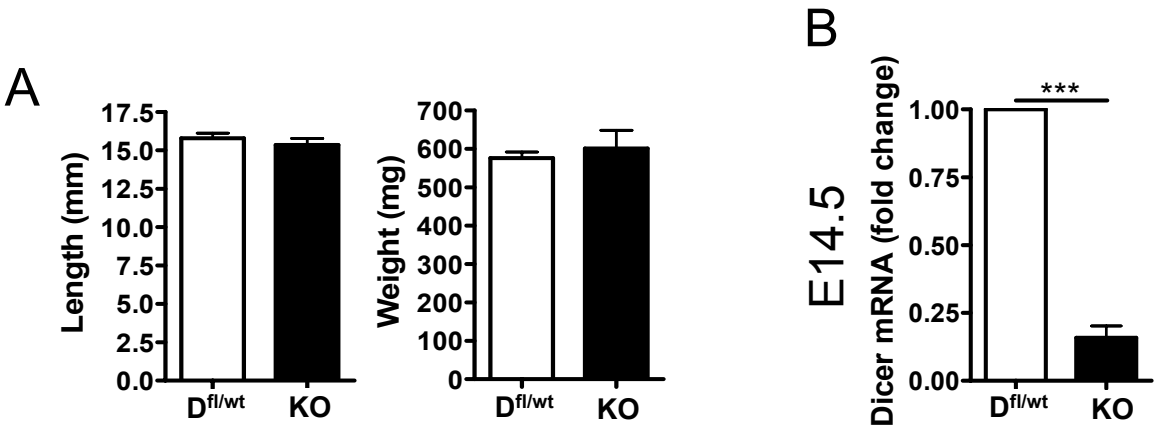
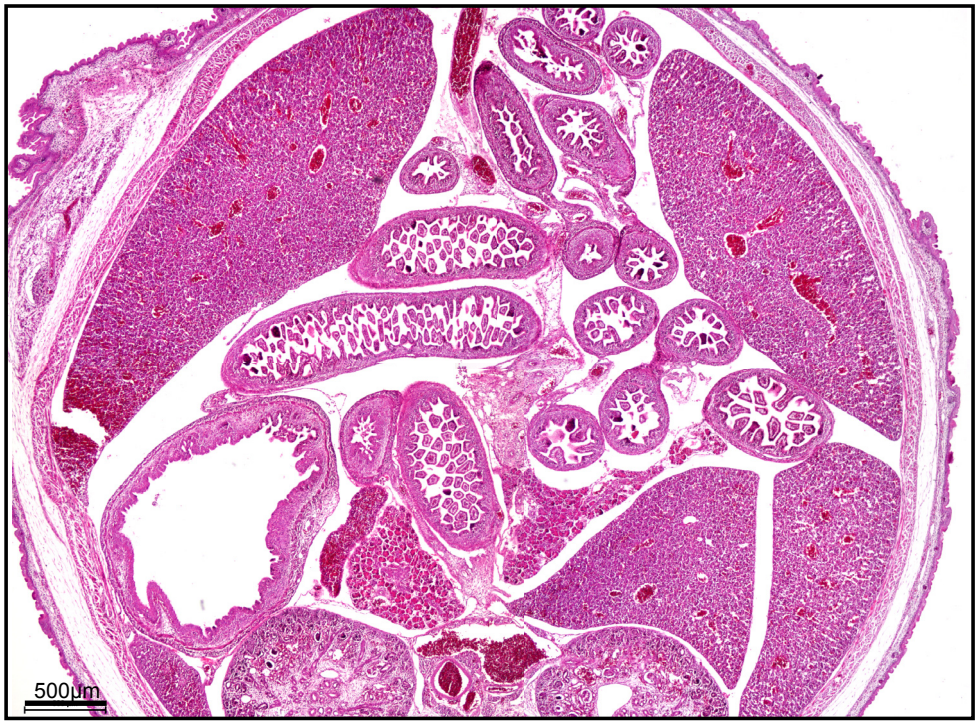
Supplemental figure I



Supplemental figure II

$D^{fl/wt}$



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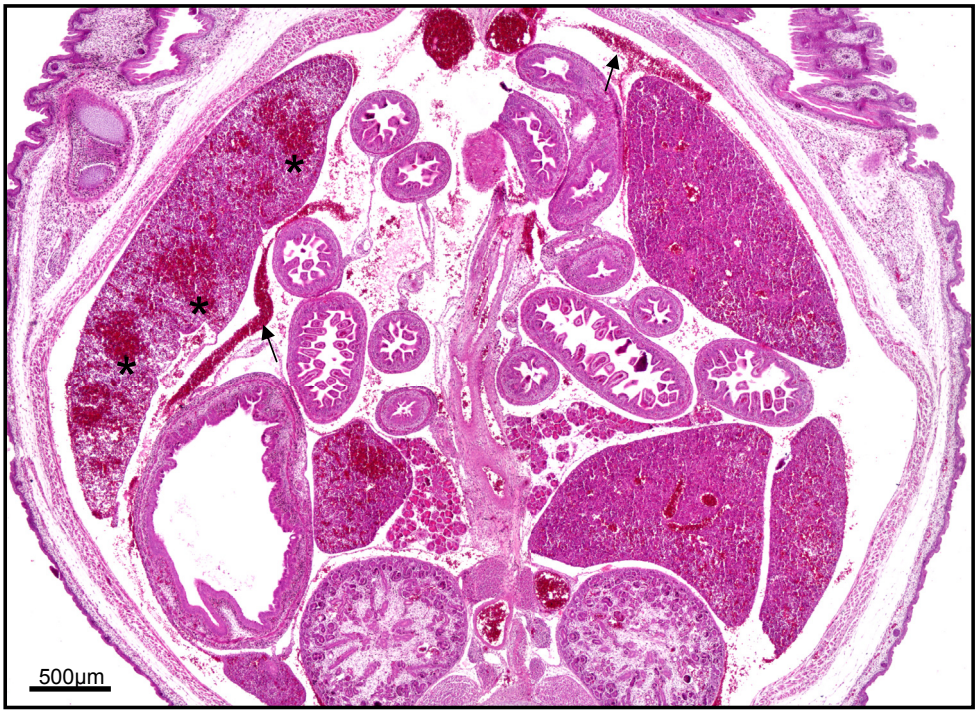

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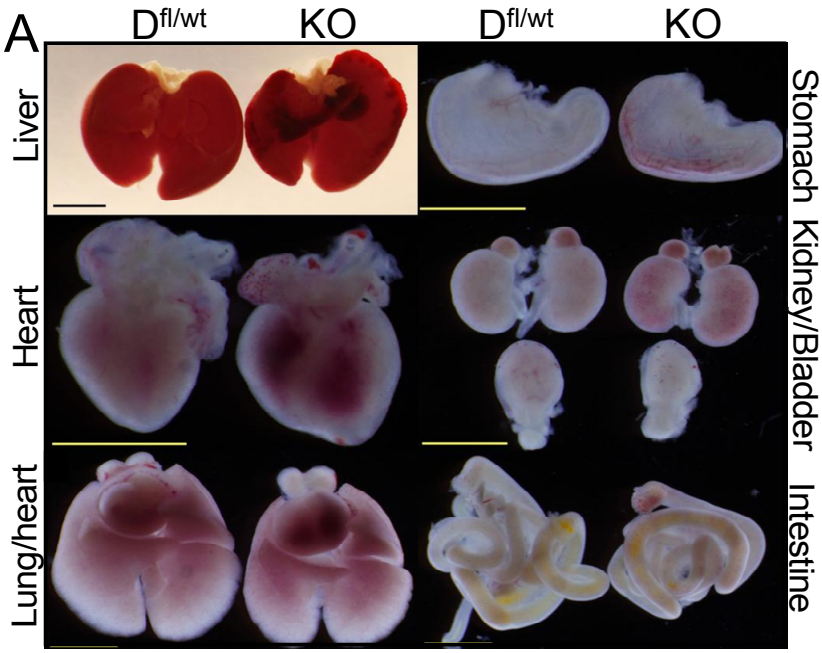

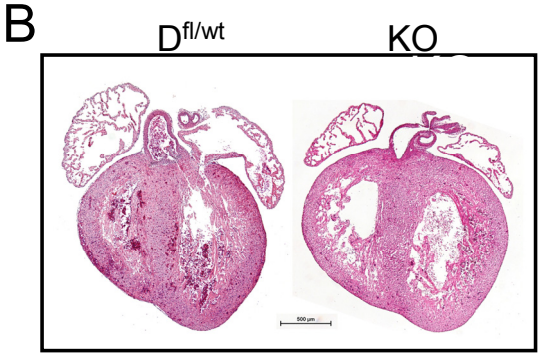

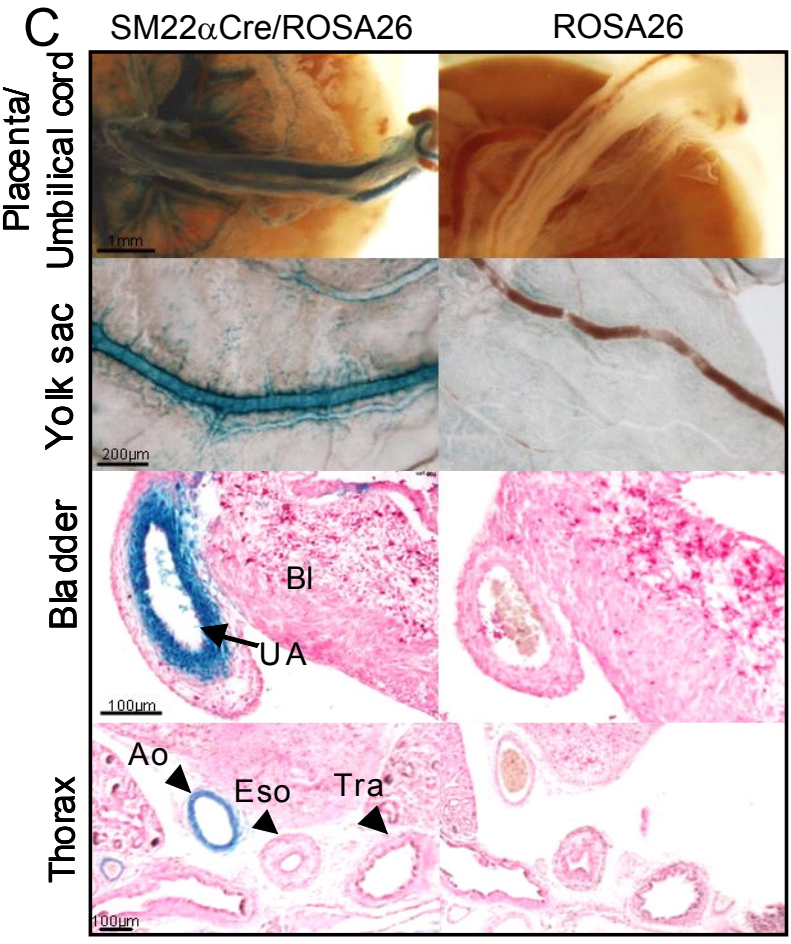

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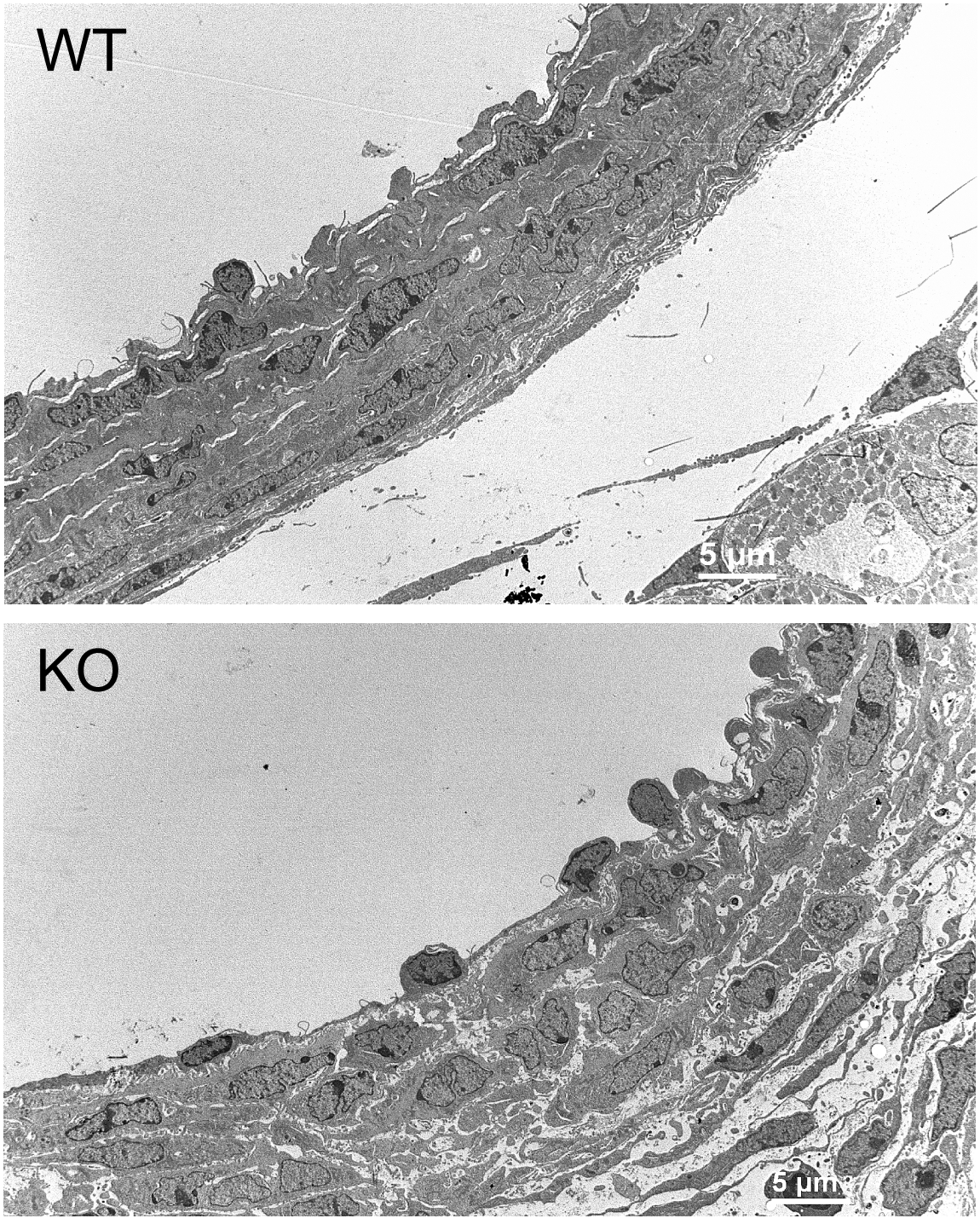

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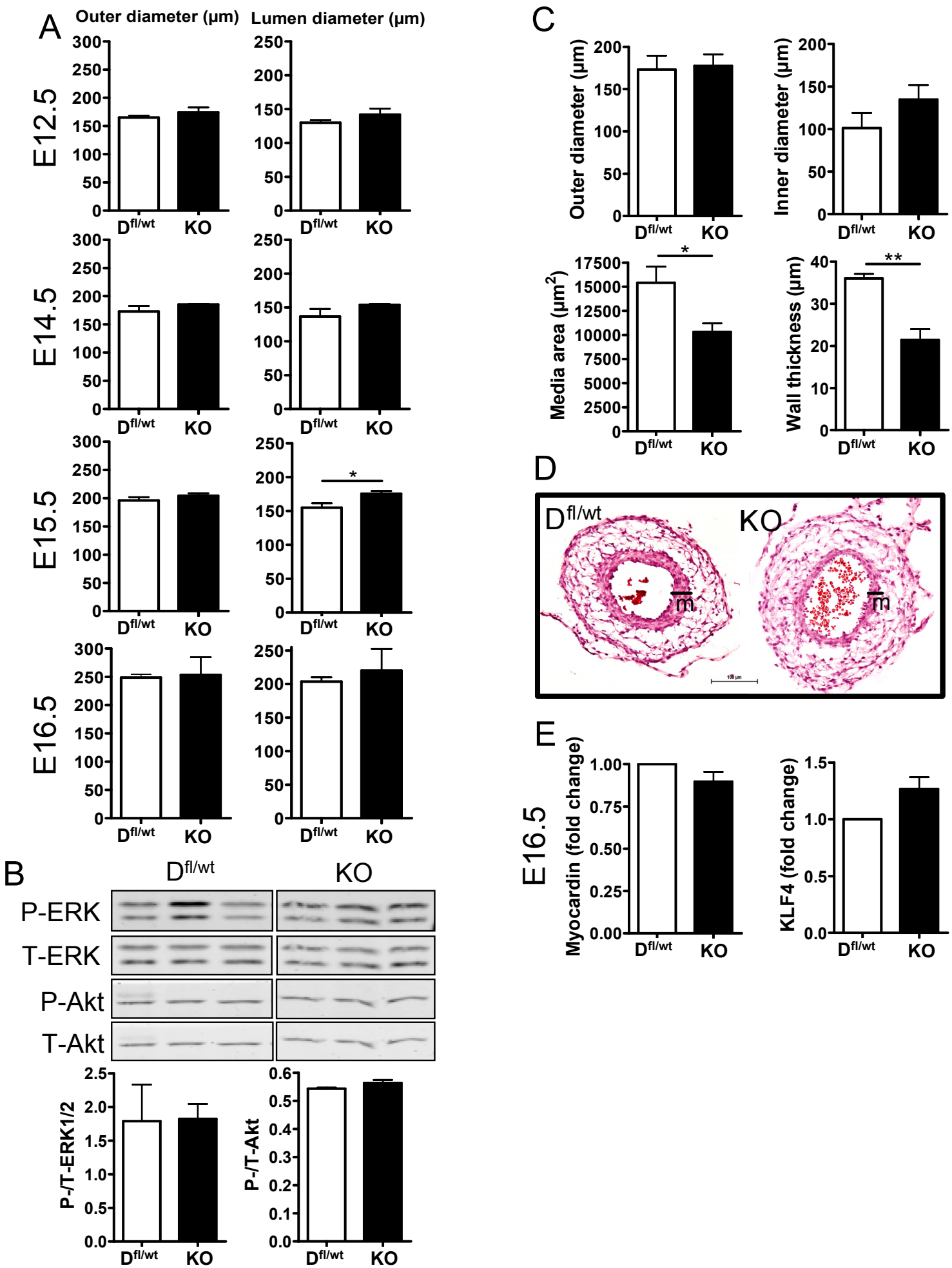

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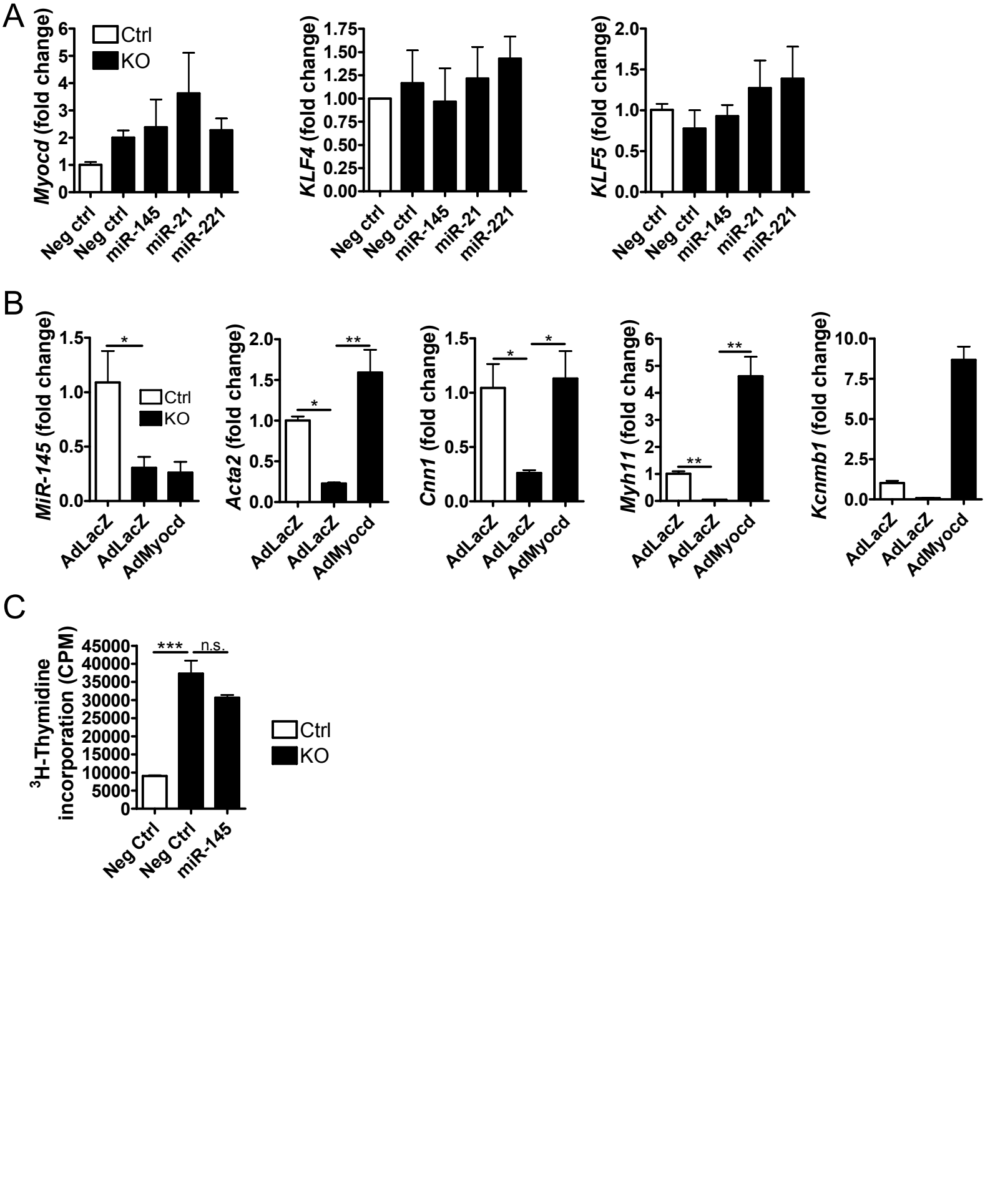

Supplemental figure VII