Autophagic Protein LC3B Confers Resistance Against Hypoxia-Induced Pulmonary Hypertension

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ONLINE DATA SUPLEMENT

Supplemental Figure legends

Fig. E1 **(A)** Mouse lung endothelial cells (MLEC) were exposed to hypoxia for 0-24 h and evaluated for expression of LC3B-I and LC3B-II by Western analysis. β-actin served as the standard. **(B)** MLEC were exposed to hypoxia for 24 h in the absence or presence of bafilomycin A1 (100 nM), and evaluated for the expression of LC3B-I and LC3B-II by Western analysis. β-actin served as the standard.

Fig. E2 (A) Representative 5 μm H&E-stained sections from *LC3B*$^{+/+}$ (*left panels*) and *LC3B*$^{-/-}$ (*right panels*) mice following normoxia (*top panels*) and 4 weeks hypoxia (*bottom panels*).
(B) *LC3B*$^{+/+}$ or *LC3B*$^{-/-}$ mice were subjected to 4 weeks hypoxia (10% O_2) or normoxia. Lung homogenates were assayed for LC3B-I and LC3B-II expression, and α-SMA expression by Western immunoblot analysis. β-actin served as the standard. No LC3B is detected in knockout animals. α-SMA expression in response to chronic hypoxia is upregulated in *LC3B*$^{-/-}$ mice. Images are presented at 100X and 200X magnification.

Fig. E3 (A) Right ventricular systolic pressure (RVSP) or (**B**) (RV/LV+S) were measured in *LC3B*$^{+/+}$ (□) and *LC3B*$^{-/-}$ (■) mice (n = 6-8) following hypoxia for 3 weeks followed by 4 weeks recovery time. **P*<0.05.

Fig. E4 **(A-C)** *Beclin 1*$^{+/+}$ (□) and *Beclin* $^{+/-}$ (■) mice were exposed to hypoxia (*n* = 15) and normoxia (*n* = 15) for 4 weeks and then measurements were taken for (A) the RVSP, and (**B**) the Fulton's index (RV/LV+S).

Fig. E5 (A-C) *Wild-type* mice were exposed to hypoxia (*n* = 15) and normoxia (*n* = 15) for 4 weeks in the absence (□) or presence (■) of daily injections of chloroquine (60 mg/kg, *i.p*) or the same volume of saline. After four weeks, measurements were taken for (A) the RVSP, (**B**) the RVW/BW ratio, and (**C**) and the Fulton's index (RV/LV+S). (D-E) Rats (*n* = 15) were injected once with monocrotaline (50 mg/kg*, s.c.*), or saline, followed by daily injections of chloroquine (20 mg/kg, *i.p.*). After two weeks, measurements were taken for (D) the RVSP, (**E**) the RVW/BW ratio, and (**F**) the Fulton's index (RV/LV+S).

Fig. E6 Lung tissue was isolated from *Egr-1*$^{+/+}$ or *Egr-1*$^{-/-}$ mice and subjected to hypoxia exposure (1% O_2) or normoxia for four weeks. Tissue sections were stained for α-smooth muscle actin.

Fig. E7 Lung fibroblasts were isolated from *Egr-1*$^{+/+}$ or *Egr-1*$^{-/-}$ mice and subjected to 24 h hypoxia exposure (1% O_2) or normoxia. Cells were fixed and stained for LC3B (red) or LAMP-1 (green). Merged images are shown at right at 40X and 100X magnification. The distribution pattern of LC3B and LAMP-1 was correlated in hypoxia-induced mouse fibroblasts (r= 0.746; P<0.01) but not in *Egr-1*$^{-/-}$ mouse fibroblast (r=0.349; NS).

Fig. E8 (A) Cultured human PAEC were exposed to hypoxia or normoxia for 24 h. Cell lysates were subjected to sucrose density gradient ultracentrifugation, to isolate 12 fractions (*see* Methods). Fractions were analyzed for the expression of caveolae marker proteins Cav-1 (Western immunoblot), or GM-1 (Dot Blot), to differentiate lipid raft fractions (fractions 3, 4, and 5) from non-raft fractions (fraction 8-12). Transferrin receptor (TfR) was used as a marker of non-rafts. Distribution of LC3B and Egr-1 is shown for full density gradient. **(B)** PAEC were transfected with GFP-LC3 (*green*) and dsRED-caveolin-1 (*red*), and then exposed to hypoxia 24 h prior to imaging. Merged images are shown at right. White arrows indicate colocalization of LC3B and caveolin-1. Data indicate loss of membrane association of LC3B with hypoxia stimulation. Analysis of individual expression values by Pearson Correlation Coefficient showed a significant positive correlation between LC3B and Cav-1 (r= 0.732; P<0.05) in normoxia. Localization of LC3B and Cav-1 (r= 0.782; P<0.01) was also significantly correlated.

Supplementary Methods

Chemicals and Reagents

We purchased the monoclonal antibody to LC3B from NanoTools, antibodies to Egr-1, caveolin-1, and LAMP-1 were from Santa Cruz; antibodies to smooth muscle actin and β-actin from Sigma; the monoclonal antibody to HIF-1α from Novus, and the antibody to PECAM from BD Biosciences. Enhanced chemiluminescence reagent was purchased from Thermo Scientific. All siRNA reagents were purchased from Santa Cruz. All other reagents were from Sigma.

Animals and *in Vivo* Exposures

All animals were housed in accordance with guidelines from the American Association for Laboratory Animal Care. The Animal Research Committee of Brigham and Women's Hospital approved all protocols. Wild-type C57BL/6 mice were placed in a Plexiglas chamber maintained at 10% O_2 (hypoxia group), or in a chamber open to room air (normoxic group), with a 12h: 12h light-dark cycle. The mice were exposed to hypoxia or normoxia for three weeks, and then the lungs were harvested and analyzed for autophagic markers by Western immunoblot analysis or for autophagosome formation by electron microscopic analysis. For functional studies, *LC3B*$^{-/-}$ mice (8-12 weeks old) (E1) or *Egr-1*$^{-/-}$ mice (Taconic, Germantown, NY) and their corresponding respective age-matched littermates were exposed to 10% O_2 (hypoxia group), or room air (normoxic group), for 4 weeks prior to functional measurements. For recovery experiments *LC3B*$^{-/-}$ mice and corresponding *LC3B*$^{+/+}$ littermates were subjected to 3 weeks hypoxia followed by 4 weeks recovery in room air, prior to functional measurements. The oxygen concentrations in the chambers were verified by an oxygen sensor calibrated for low oxygen tension.

Right Ventricular Systolic Pressure and Heart Weight Measurements

Measurements were obtained using a PC-driven PowerLab (ADInstruments, Colorado Springs, CO). The system was calibrated before each experiment. Each mouse was weighed and injected with sodium pentobarbital (60 mg/kg) to induce anesthesia with maintenance of spontaneous respiration. The abdomen was opened and the left and right ventricles visualized through an intact diaphragm. A 23 G needle was

then inserted into the right ventricle and the RVP recorded. The diaphragm was then opened, and then a repeat measurement of the RVP as well as LVP was obtained. The right ventricular (RV) free wall was dissected and weighed separately from the left ventricle and septum (LV + S), which were weighed together.

Vascular Cell Culture

Human pulmonary artery endothelial cells (PAEC) were purchased from Lonza. PAEC at passages 5-8 were grown to ~80% confluence in endothelial cell growth medium-2 (EGM-2) supplemented with EGM-2 SingleQuots™ and 2% FBS (Lonza). Human pulmonary artery vascular smooth muscle cells (PASMC)(Lonza) at passages 7-10 were grown to ~80% confluence in smooth muscle cell growth medium-2 (SmGM2) supplemented with SmGM2 SingleQuots™ and 5% FBS (Lonza). Cells were cultured in humidified incubators containing 95% air, 5% CO_2 at 37^{o}C. Cells were grown in 100 mm dishes and detached with 0.05% trypsin, resuspended in complete growth medium, and seeded into 35 mm, 6 well, and 12 well plates for individual experiments. Cells were subjected to transient transfection with either siRNA or expression vectors to modulate the cellular expression of LC3B or Egr-1. For proliferation experiments, transiently transfected cells at 80% confluence were starved for 2 h in serum free EGM-2 media prior to the addition of PDGF-BB (20 ng ml^{-1}) or ET-1 (40 nM) in complete growth media, and then placed in hypoxia or normoxia for an additional 48 h.

For hypoxic exposures, PAEC or PASMC were placed in an airtight Modular Incubator Chamber (Billups-Rothenberg), flushed continuously (10 min) with a premixed gas (1% O_2, 5% CO_2, 94% N_2) and then incubated for the indicated intervals. Corresponding normoxic controls were maintained for equivalent times in humidified incubators in an atmosphere of 95% air, 5% CO_2. Cell proliferation was assessed for proliferation using a standard MTT assay and cell counting, or for the expression of specific proteins by Western immunoblot analysis.

Transient Transfection

Human PAEC and PASMC were transfected with human *LC3B*-siRNA, and control-siRNA as described by the manufacturer (Santa Cruz). PAEC at passages 6-8 were seeded at $5x10^4$ cells per well in 12 well dishes. After 24 h each well was approximately 80-90% confluent. The media was changed to transfection media (Santa Cruz) for 2 h. 10 nM siRNA was incubated with transfection reagent (Santa Cruz) for 1 h and then added to each well. After 4-6 h, the media was aspirated and complete media replaced in each well. Treatment with hypoxia was initiated 24 to 48 h post-transfection.

For overexpression experiments, LC3B was subcloned into pCMV vectors (Origene). PAECs and PASMCs were transiently transfected for 4 h in 24-well dishes with 1.5 μg of the empty vector (pCMV) or vector containing the gene for LC3B using LipofectAMINE plus (Life Technologies), according to the manufacturer's instructions. The cells were then supplemented with DMEM with 10% serum and cultured for 36 h, followed by Western blotting to confirm protein expression.

To examine the distribution of GFP-LC3B, 1 μg of GFP-LC3B (rat), a kind gift of Dr. Noboru Mizushima (Tokyo Medical and Dental University) was transfected into 2 x 10^5 cells using lipofectamine^{TM}2000 according to the supplier's protocol (Invitrogen, CA).

Electron Microscopy and Imaging

For electron microscopy, tissue section or cells were fixed in 2.5% glutaraldehyde in PBS after experimental manipulations. These tissues or cells were photographed using a JEOL JEM 1210 transmission electron microscope (JEOL) at 80 or 60 kV onto electron microscope film (ESTAR thick base; Kodak) and printed onto photographic paper. For microscopy, lung tissue sections were fixed in formalin and embedded in paraffin. Confocal imaging was performed on an LSM510 meta inverted microscope equipped with an oil immersion lens at 40X objective, with a confocal laser scanning head (Carl Zeiss). Digital images were acquired for analysis (SPOT, Diagnostic Instruments, Inc.).

Western Immunoblotting and Immunohistochemistry

Western immunoblot analyses, co-IP, and immunohistochemical staining were performed as previously described (E2). For Western blotting, protein concentrations of cell lysates and frozen tissue homogenates were determined using Bradford reagent (BioRad) and were resolved by SDS/PAGE using NuPage Novex Bis-Tris 4-12% polyacrylamide gels (Invitrogen). Formalin-fixed lung tissue sections were paraffin-embedded for immunohistochemical staining.

Colocalization between LC3B and LAMP-1 was examined by an immunocytochemical method. Briefly, cells were fixed with 2% paraformaldehyde and permeabilized with 0.2% Triton X-100. After washing with PBS, the slides were blocked with 3% bovine serum albumin for 1 h and incubated with rat monoclonal anti-LAMP-1 antibody (1:100, Santa Cruz) and rabbit polyclonal anti-LC3B antibody (1:100, Sigma) for 3 h at 4^{o}C. The cells were washed and incubated with anti-rat IgG-FITC and anti-rabbit IgG-TRITC for 1 h. After staining, the cells were mounted with mounting medium and observed by laser scanning confocal microscopy (Carl Zeiss).

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) assays were performed as previously described (E3). The primer set 5-GCTCGGGACAAAAGCAGTT-3′and 5′-CCCTGAGGTGACGGTTGT-3′was used to amplify a 427 bp DNA fragment (-550/-124) of the *LC3B* promoter region. After treatment with hypoxia, PAEC were harvested and subjected to the ChIP assay (Active Motif), using the manufacturer's protocol.

Lipid Raft Isolation

Lipid raft fractions were isolated by sucrose gradient ultra-centrifugation as described, but in the absence of detergent.[2] Cells were lysed in ice-cold MBS buffer (25 mM MES, pH 6.5, 150 mM NaCl, 1 mM Na_3VO_4, and protease inhibitors). Lysates were adjusted to 4 ml of 40% sucrose by the addition 2 ml of 80% sucrose, 4 ml of 35% sucrose, 4 ml of 5% sucrose in MBS buffer. Samples were ultra-centrifuged at 39,000 x *g* for 18 h and fractionated into 12 subfractions.

To validate the fractionation method, fractions were analyzed for organelle marker proteins by Western blotting. The transferrin receptor, a marker for non-caveolae plasma membrane was well separated from the major caveolae fractions, indicating that non-caveolae plasma membrane did not significantly contaminate the major caveolae fractions. Caveolin-1 and GM-1, positive markers for caveolae were detected from the caveolae fraction. We also measured Grp78, a marker for ER, cytochrome C, a marker for mitochondria, and Golgin-97, a marker for Golgi, which are compartmentalized to the higher density fractions.

Real-Time PCR

Total RNA was extracted from cells using TRIZOL reagent (Invitrogen), and converted to cDNA using high-capacity cDNA archive kit (Applied Biosystems). Quantitative RT-PCR was performed as described (E4). Primers for LC3B and TaqMan Master Mix for gene expression assays were purchased from Applied Biosystems. Gene expression was analyzed by the comparative threshold cycle (Ct) method, using 18S rRNA as the internal standards.

Histologic Analysis and Morphometry Lungs were inflated, harvested, fixed in 2% paraformaldehyde, and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E)(E5). Remodeling was quantitated as described previously (E6-E8). Percent wall thickness was calculated as: wall thickness (%) = $(area_{ext} - area_{int}) \div area_{ext} \times 100$, where $area_{ext}$ represents the external diameter and $area_{int}$ represents the internal diameter of each vessel respectively (E6-E8).

Measurement of Reactive Oxygen Species

The ROS level in cultured hPAEC was measured *in situ* by using 2′,7′-dichlorohydrofluorescin diacetate (H_2DCF-DA) (Invitrogen). Briefly, hPAECs were treated with hypoxia (1%) exposure for 30 min and incubated with 10 μM H_2DCF-DA for another 30 min at 37^{o}C. After the excess probe was removed, cells were incubated for an additional 20 min to allow complete de-esterification of the intracellular diacetates. The fluorescence images of at least 10 randomly selected cells per dish were captured using a confocal laser microscope.

Statistics

Data are presented as mean ± s.d. Paired analysis was performed with the Student *t*-test as appropriate. Parametric analyses were carried out by one-way ANOVA with Tukey's Method. Correlations between parameters were expressed with the Pearson correlation coefficient (*r*). Statistical significance was accepted at *; $P<0.05$, #; $P<0.01$.

Supplementary References

E1. Cann GM, et al. (2008) Developmental expression of LC3alpha and beta: absence of fibronectin or autophagy phenotype in LC3beta knockout mice. *Dev Dyn* 237: 187-195.

E2. Kim HP, Wang X, Galbiati F, Ryter SW, Choi AM. (2004) Caveolae compartmentalization of heme oxygenase-1 in endothelial cells. *FASEB J* 18: 1080-1099.

E3. Chen, ZH, *et al.* (2008) Egr-1 regulates autophagy in cigarette smoke-induced chronic obstructive pulmonary disease. *PLoS ONE* 3: e3316.

E4. Slebos DJ, *et al.* (2007) Mitochondrial localization and function of heme oxygenase-1 in cigarette smoke-induced cell death. *Am J Respir Cell Mol Biol* 36: 409-417.

E5. Fredenburgh LE, *et al.* (2008) Absence of cyclooxygenase-2 exacerbates hypoxia-induced pulmonary hypertension and enhances contractility of vascular smooth muscle cells. *Circulation* 117: 2114–2122.

E6. Christou H, *et al.* (2000) Prevention of hypoxia-induced pulmonary hypertension by enhancement of endogenous heme oxygenase-1 in the rat. *Circ Res* 86: 1224–1229.

E7. Minamino T, *et al.* (2001) Targeted expression of heme oxygenase-1 prevents the pulmonary inflammatory and vascular responses to hypoxia. *Proc Natl Acad Sci* 98: 8798–8803.

E8. Yet SF, *et al.* Hypoxia induces severe right ventricular dilatation and infarction in heme oxygenase-1 null mice. *J Clin Invest* 103: R23–R29.

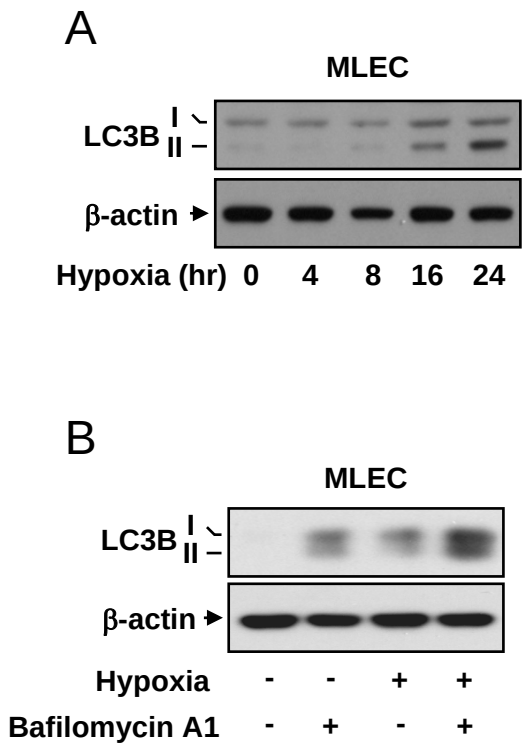
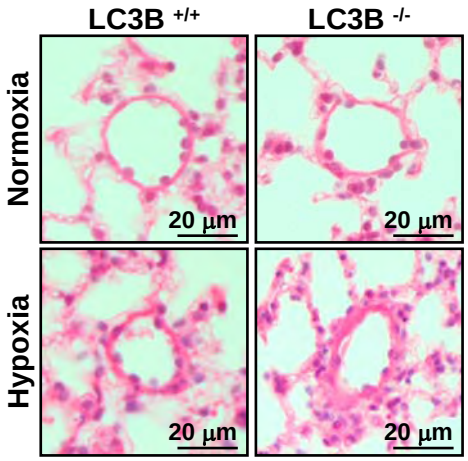

A
MLEC
LC3B I
II
β-actin
Hypoxia (hr) 0 4 8 16 24
B
MLEC
LC3B I
II
β-actin
Hypoxia - - + +
Bafilomycin A1 - + - +

Figure E1

A


LC3B +/+
LC3B -/-
Normoxia
Hypoxia
20 μm
20 μm
20 μm
20 μm

B

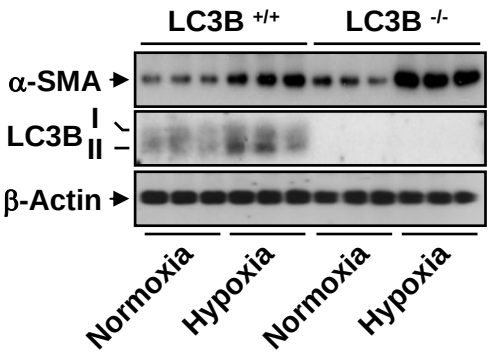

LC3B +/+
LC3B -/-
α-SMA
LC3B I
II
β-Actin
Normoxia
Hypoxia
Normoxia
Hypoxia

Figure E2

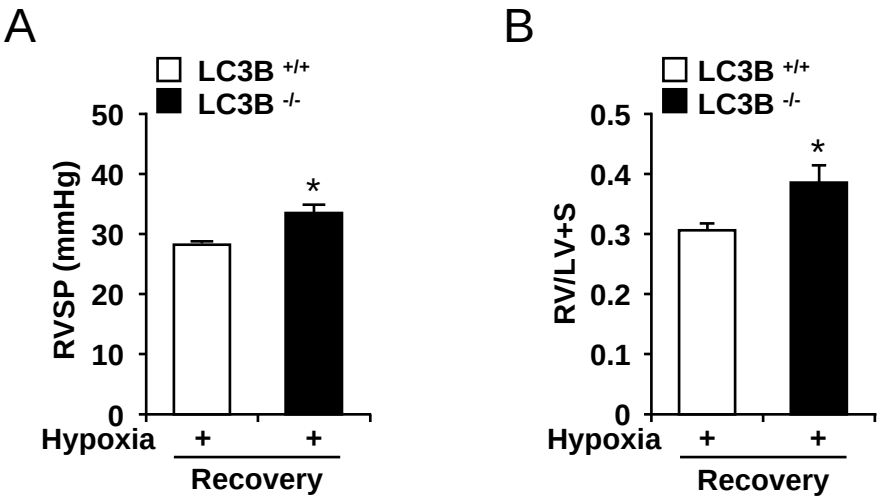

A
LC3B +/+
LC3B -/-
RVSP (mmHg)
50
40
30
20
10
0
*
Hypoxia + +
Recovery
B
LC3B +/+
LC3B -/-
RV/LV+S
0.5
0.4
0.3
0.2
0.1
0
*
Hypoxia + +
Recovery

Figure E3

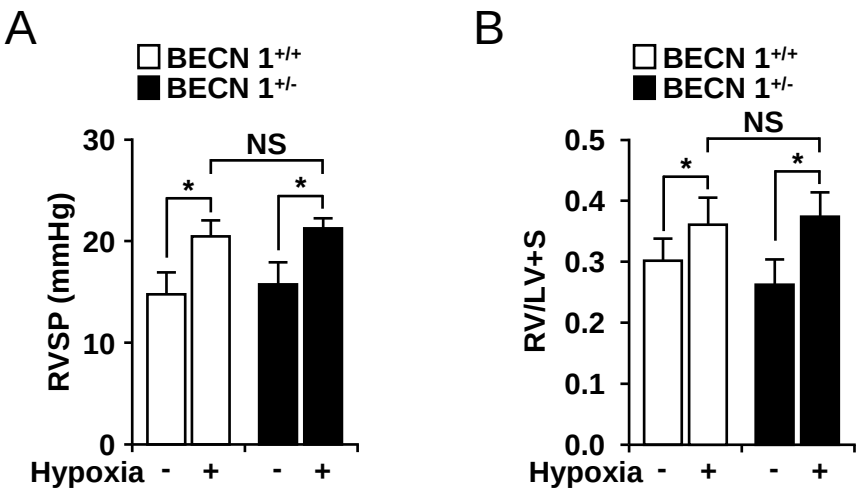

A
BECN 1+/+
BECN 1+/-
RVSP (mmHg)
30
20
10
0
NS
*
*
Hypoxia - + - +
B
BECN 1+/+
BECN 1+/-
RV/LV+S
0.5
0.4
0.3
0.2
0.1
0.0
NS
*
*
Hypoxia - + - +

Figure E4

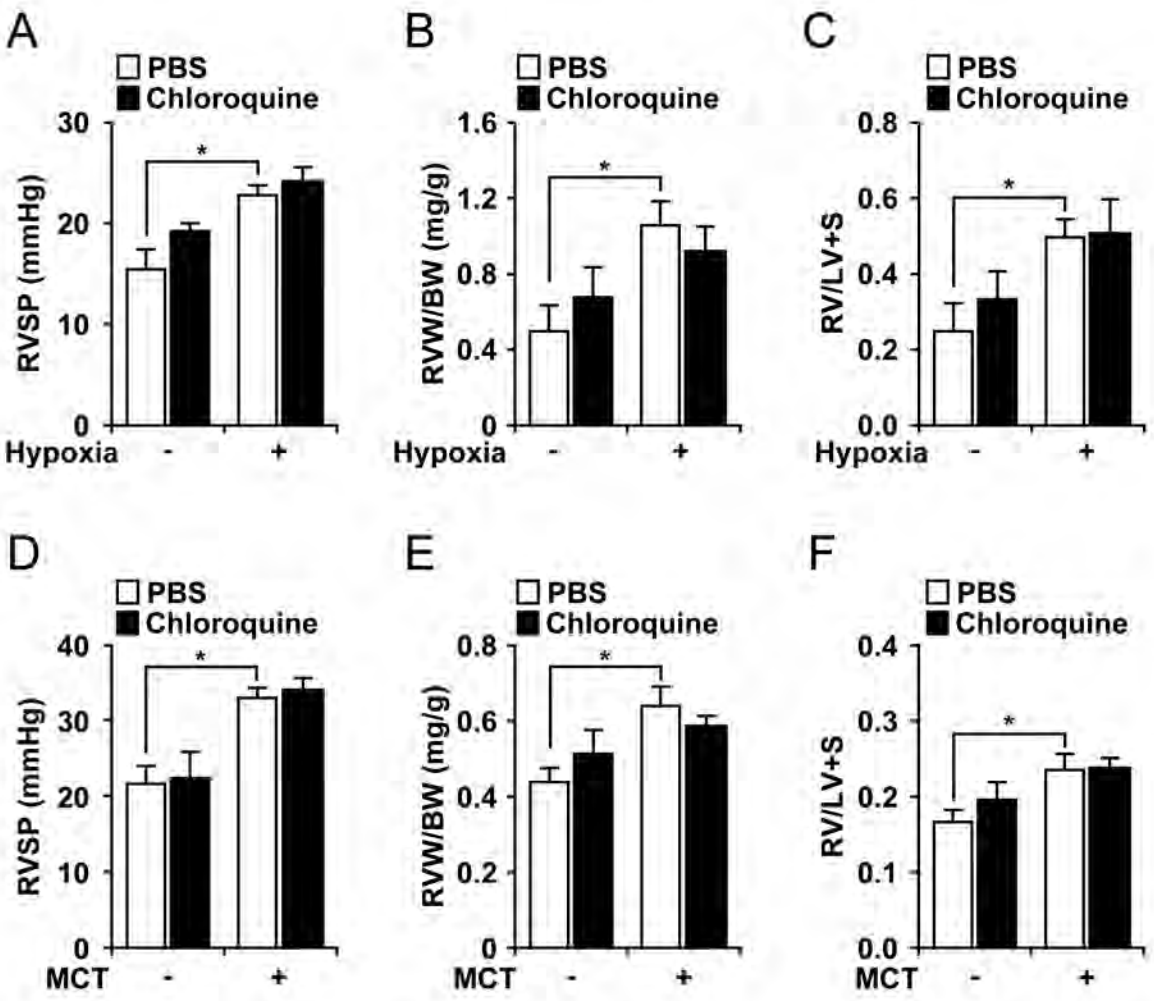

A
PBS
Chloroquine
RVSP (mmHg)
30
20
10
0
*
Hypoxia - +
B
PBS
Chloroquine
RVW/BW (mg/g)
1.6
1.2
0.8
0.4
0
*
Hypoxia - +
C
PBS
Chloroquine
RV/LV+S
0.8
0.6
0.4
0.2
0.0
*
Hypoxia - +
D
PBS
Chloroquine
RVSP (mmHg)
40
30
20
10
0
*
MCT - +
E
PBS
Chloroquine
RVW/BW (mg/g)
0.8
0.6
0.4
0.2
0
*
MCT - +
F
PBS
Chloroquine
RV/LV+S
0.4
0.3
0.2
0.1
0.0
*
MCT - +

Figure E5

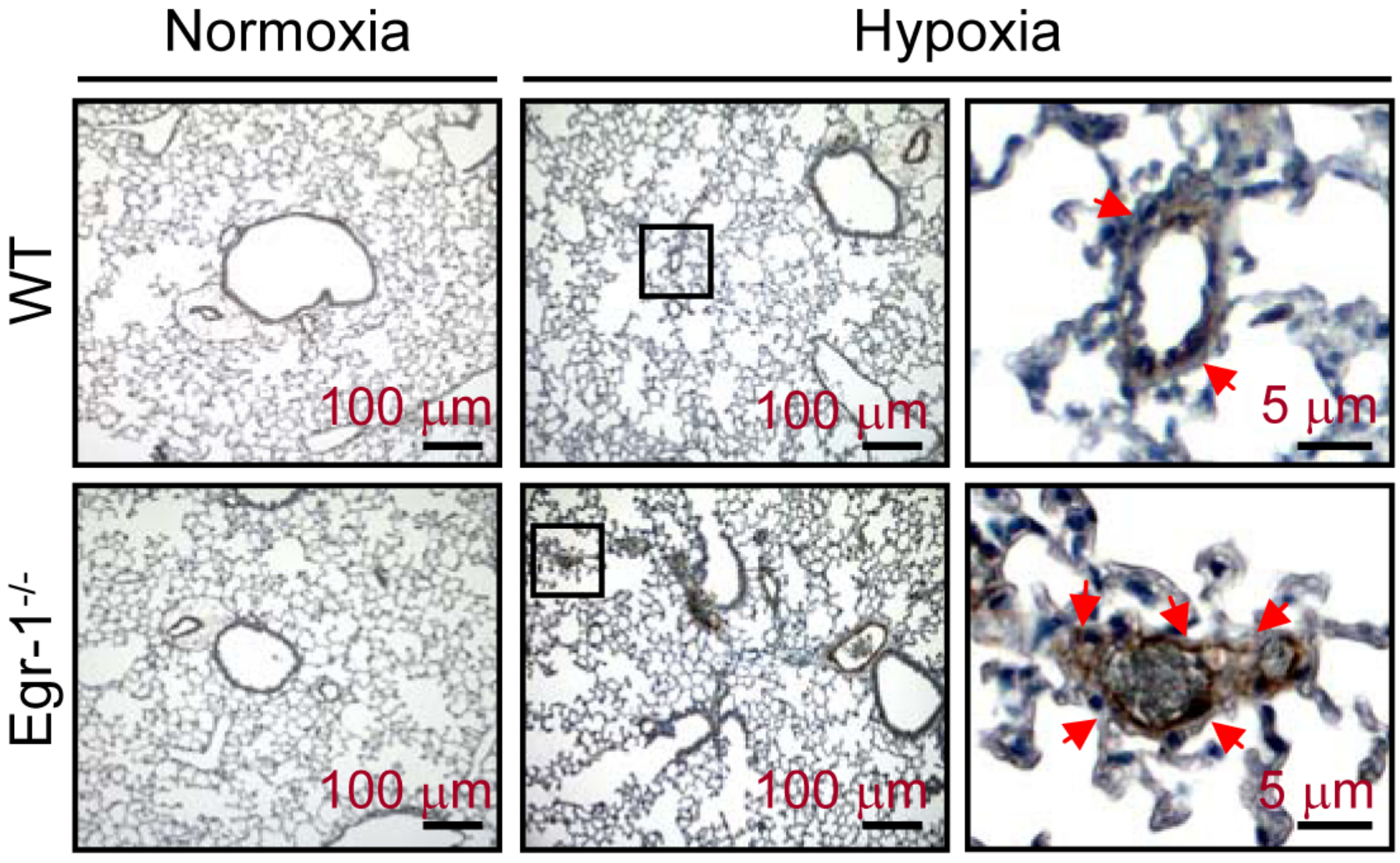


Figure E6

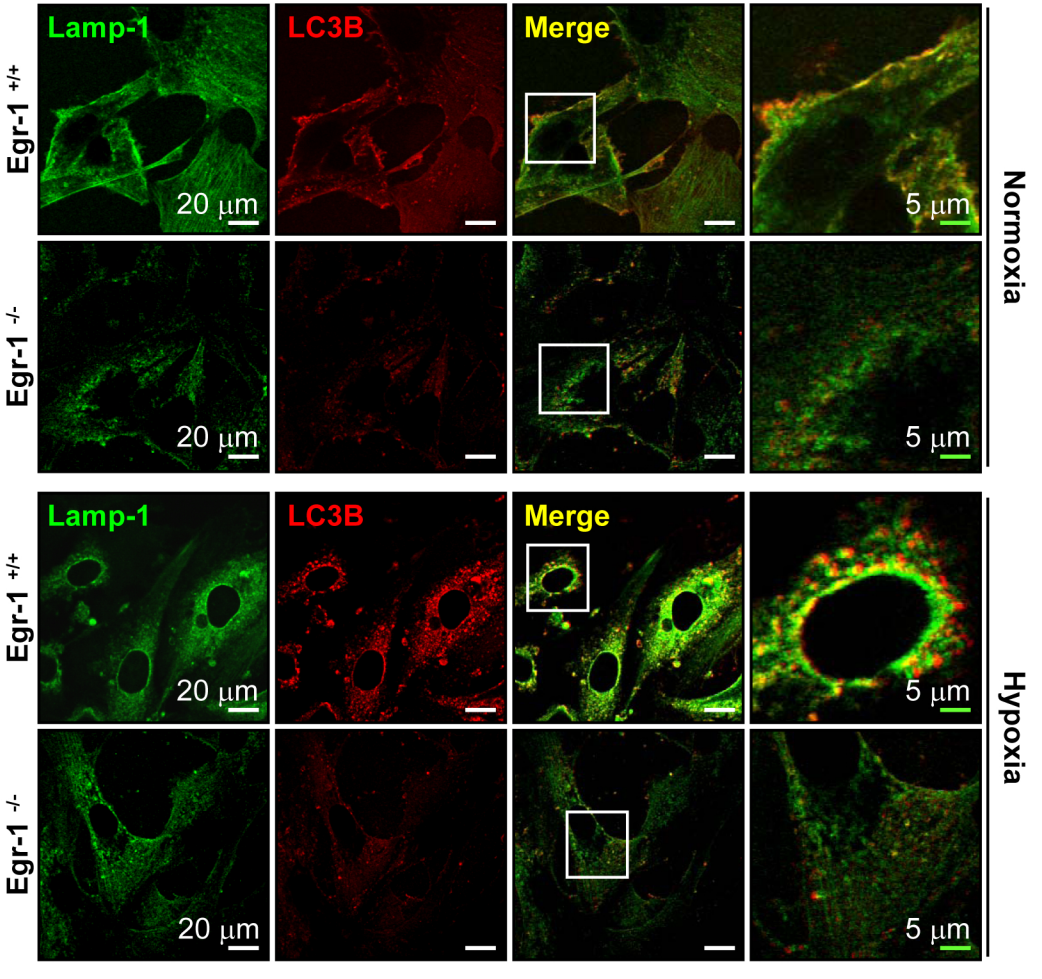
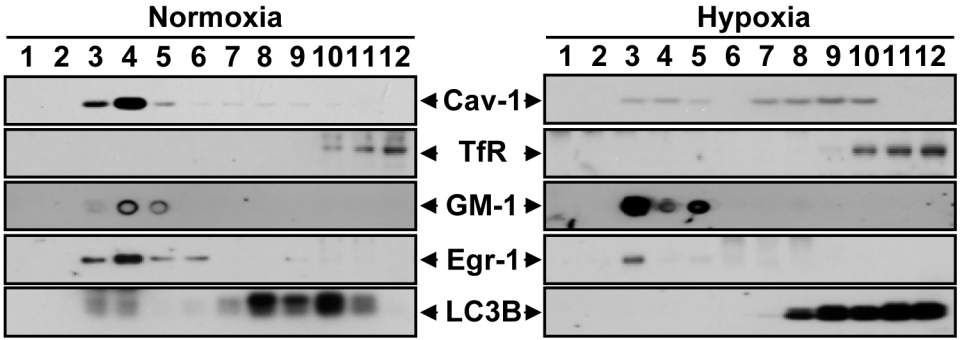


Figure E7

A


Normoxia
1 2 3 4 5 6 7 8 9 10 11 12
Hypoxia
1 2 3 4 5 6 7 8 9 10 11 12
Cav-1
TfR
GM-1
Egr-1
LC3B

B

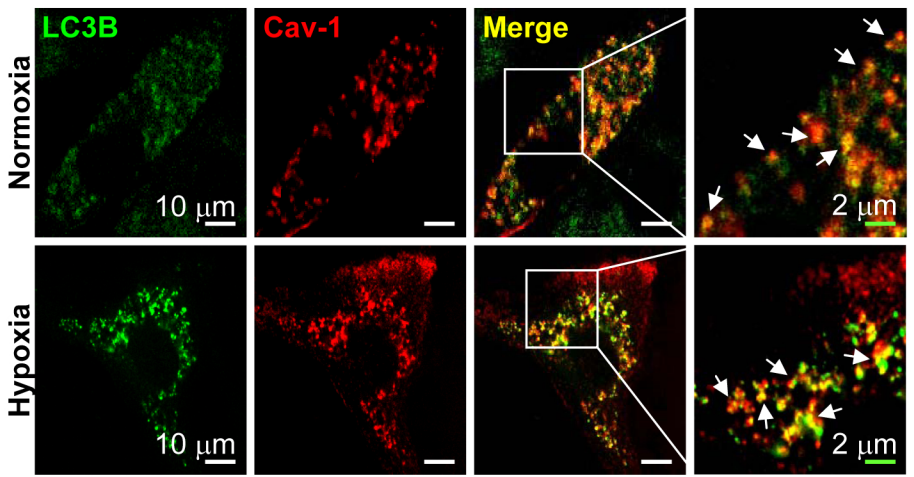

LC3B
Cav-1
Merge
Normoxia
Hypoxia
10 μm
10 μm
2 μm
2 μm

Figure E8