SUPPLEMENTAL MATERIAL

Supplemental Methods:

Targeted disruption of GIT1

Transformed *E. coli* stocks containing five of the BAC constructs that hybridized to the GIT1 cDNA were purchased (BACPAC resources). The GIT1 gene was subcloned into the Xba I site of pBlueScript II. Following cloning into pBlueScript II, a gene targeting vector replacing exons 2-7 with a neo cassette was created for ubiquitous deletion of GIT1 expression. The 6 kb (exon2-exon7) Xba I fragment of the GIT1 gene was released from pBluescript II, and the 8 kb fragment, was released from its vector by digestion with BsaB I and Xba I and inserted into the targeting vector at the Swa I/Avr II site.

For creation of knockout mice, linearized targeting vector was electroporated into mouse strain 129 embryonic stem (ES) cells. Drug selection for cells with incorporation of the targeting vector by homologous recombination was performed with G418. Surviving cell colonies were then screened for proper recombination by Southern blot. The ES cells were screened by digestion with Xba I, then hybridization of a radiolabeled probe. The Southern blot results were confirmed by PCR screening of the ES cell colonies. Colonies were tested for the presence of the neo cassette at the proper locus by using the primers 5'- GAT CAA TTC TCT AGC TAG AGC TCG CTG A -3', which binds within the neo cassette, and 5'- ACC TCT GTA CCC TAC CAC CTC TTG -3', which binds to intron 7 near the neo cassette. ES cell lines determined to possess the properly recombined targeting vector were injected into C57/Bl6 blastocysts and implanted into pseudo-pregnant mothers. Offspring were screened for the presence of recombinant tissue by PCR using the protocol for screening ES cell colonies. All mice found to have recombinant tissue by PCR also demonstrated chimeric coat color. Chimeric mice were backcrossed with

C57/Bl6 mice (>4 generations). Offspring were genotyped by PCR. To further confirm the knockout of GIT1 gene and assess compensation by GIT2, GIT1 and GIT2 mRNA expression were assayed by RT-PCR. Briefly total lung mRNA from WT and KO mice of P4 was obtained using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Then the RNA (1 μg) was reverse transcribed using random primers and Superscript II RT. The single-strand cDNA was amplified by PCR with *Taq* DNA polymerase. The sequences of sense and antisense primers, corresponding PCR conditions, and cycle counts were as follows: GIT1 (exon1-4): 5'-ACGAACGCCAGCATCTGGTACTTT-3' and 5'- ATCAGCAGAGGTGTGCTGGTTTGT-3'; GIT1(exon3-13): 5'-AACTTGGAAACATGTCTGCGCCTG-3' and 5'-TGGCAAACTCTCGGGCATTGAAAC-3'; GIT1(exon7-19): 5'-ACATCATCCCACAGATGGCTGACA -3' and 5'-GTGGCAAACTCTCGGGCATTGAAA -3'; and GIT2: 5'- TATGACAGTGTGGCCTCCGATGAA-3' and 5'-AGGGTCACAGGCAGTGTTGTCATA-3', 45 s at 94°C, 45 s at 55°C, and 45 s at 72°C, 35 cycles. GAPDH cDNA was amplified as a control.

Blood parameter assay

GIT1 WT and KO mice (n=3 per group) were anesthetized with an intraperitoneal injection of ketamine (130 mg/kg) and xylazine (8.8 mg/kg) in saline (10 mL/kg). Then tail snipping was performed aseptically and 20 ul blood/per mice were obtained by gently 'milking' the tail. Blood parameters were assayed by hematology analyzer veterinary (HESKA).

Immunohistochemistry

For cryosections, the lungs of WT mice (P5) were inflated with Tissue-tek (Sakura Fine Technical Co, Ltd., Tokyo, Japan) in 0.9% NaCl (1:1) and embedded. The cryosections were blocked with 10% normal goat serum/PBS for 30 min. PECAM1 antibody (BD Pharmingen,

1:400) or GIT1(Santa Cruz, 1:400) were incubated for 2h at room temperature. After washing three times with PBS, sections were incubated with Alexa Fluor 488 goat anti-rat antibody or Alexa Fluor 546 conjugated donkey anti goat antibody (Invitrogen, 1:400) for 30 min at room temperature. Sections were washed three times with PBS and then mounted in the Vecta shield (Vector Laboratory, South San Francisco, CA).

Vessel density was evaluated using rat anti-mouse vWF (DAKO) monoclonal antibody, with hematoxylin counterstain. A high temperature (120°C) under pressure was used for antigen retrieval with 10 mmol/L citrate buffer (pH 6.0) for 20 minutes. Primary antibodies were incubated at 4°C overnight, followed by incubation with the secondary antibody for 30 minutes and ABC complex for 30 minutes. The peroxidase-binding sites were verified by the 3,3' diaminobenzidine (DAB) in chromogen solution (DakoCytomation Inc).

Paraffin sections were incubated with primary antibody overnight at 4ºC. The primary antibodies used were surfactant protein C (SP-C) polyclonal (1:50 dilution; Santa Cruz), smooth muscle α-actin (αSMA, clone 1A4, 1:400 dilution; Sigma-Aldrich). For antigen retrieval of SP-C antibodies, sections were heated with 10 mmol/L citrate buffer (pH 6.0) for 20 minutes at 120ºC. Subsequently, the sections were incubated with FITC conjugated goat anti-rabbit (Vector Labs, FI-1200) secondary antibodies for 1h at room temperature, and mounted with Vecta Shield with DAPI (1:10). For detection of αSMA antigens, ARK™ (Animal Research kit, DakoCytomation) was used. As a negative control, species- and isotype-matched IgG were used in place of the primary antibody. Slides were viewed with a microscope (BX41, Olympus) and with digital camera (Spot Insight 2, Diagnostic Instruments, Inc.).

RT-PCR assay

Total lung mRNA from WT and KO mice of 2-3 month (n=3 per group) was obtained as

described above. The sequences of sense and antisense primers, corresponding PCR conditions, and cycle counts were as follows: Endothelin-1 (ET-1): 5'- GCC ATC AGC AAT AGC ATC AAG GCA -3' and 5'- TCC TCT GCC CGT CTG AAC AAG AAA -3'; Endothelin-1 receptor A(ET-A): 5'- ATC ACC GTC TTG AAC CTC TGT GCT -3' and 5'- TCC TAT GCA GCT CGC CCT TGT ATT -3'; Fibroblast growth factors10(FGF-10): 5'- TAC TGA CAC ATT GTG CCT CAG CCT -3' and 5'- AGT TGC TGT TGA TGG CTT TGA CGG -3'; 45 s at 94°C, 45 s at 60°C, and 1min at 72°C, 35 cycles. β-actin cDNA was amplified as a control.

Real-Time PCR Assay

Total lung mRNA from WT and KO mice (P5, n=3 per group) was obtained. cDNA was amplified with a qPCR kit with SYBR Green (Life Technologies Inc). The sequences of primers were as following: VEGF: 5'- TCA CCA AAG CCA GCA CAT AGG AGA -3' and 5'- TTT CTC CGC TCT GAA CAA GGC TCA -3'. ß-actin: 5'- TGT GAT GGT GGG AAT GGG TCA GAA-3, 5'- TGT GGT GCC AGA TCT TCT CCA TGT-3. Data were calculated by the $2\Delta\Delta^{-CT}$ method and presented as fold change of VEGF relative to ß-actin.

Supplemental figure legends

Supplemental Fig. 1. HE staining of adult GIT1 WT and KO mice

Hematoxylin and eosin (H&E) staining of lung sections from GIT1 WT and KO mice (2-3 month old). GIT1 WT mice show normal, well-developed saccular and alveolar airway structures (**A, C**), whereas GIT1 KO showed abnormally large airspaces (**B, D**). Bar=50 μm

Supplemental Fig. 2. GIT1 KO mice show normal tight junctions in lung endothelial cells

A. Electron micrographs of GIT1 WT lung endothelial cells show intact tight junctions in capillary. **B-D.** The tight junctions in endothelial cells in capillaries, arterioles and venules from GIT1 KO mice are normal.

Supplemental Fig. 3. Colocalization of GIT1 and PECAM-1 in neonatal GIT1 WT mice (P5)

Immunostaining of GIT1 and PECAM-1 were performed on frozen lung sections of GIT1 WT mice (P5). GIT1 colocalized with PECAM-1, especially in capillaries (**D-F**). GIT1 was also expressed in smooth muscle cells and bronchi (**A-C**).

Supplemental Fig. 4. Smooth muscle α-actin(αSMA) expression in the lungs of GIT1 WT and KO mice (P5)

αSMA was stained in lungs of GIT1 WT (**A**) and KO (**B**) at P7. Brown staining indicates positive cells. Bar=50 μm **C**. Quantification of αSMA staining. 5 fields were chosen from each sample (n=3 per group).

Supplemental Fig. 5. Immunostaining of SP-C in the lung of GIT1 WT and KO mice (P7)

SP-C staining of lungs from GIT1 WT (**A**) and KO (**B**) at P7. Green fluorescence staining is SP-C positive cells and nuclei were counterstained by DAPI. **C.** Quantification of SP-C positive cells (*P<0.05, n=3 per group). 5 fields were chosen from each sample. **D**. Ratio of SP-C positive cells and total cells (n=3 per group). Bar=50 μm

Supplemental Fig. 6. RT-PCR analysis of ET-1, ET-A receptor, and FGF-10 mRNA levels in GIT1 WT and KO mice

Total RNA from lung tissues of GIT1 WT, KO mice (2-3 months) was reverse transcribed, and cDNA was amplified using PCR primers specific for ET-1, ET-A receptor, FGF-10, GIT1 and β-Actin. The ET-1, ET-A, and FGF-10 ratios relative to actin for WT mice were set to 1.0. Data represent means ± SE of 3 different experiments.

Supplemental Fig. 7. mRNA expression of VEGF was decreased in GIT1 KO mice.

Total RNA from lung tissues of GIT1 WT and KO mice (P5) was reverse transcribed, and VEGF and ß-actin mRNA expression were measured by real-time PCR. The VEGF to- ß-actin ratios for WT mice were set to 100%. Data represent means ± SE of 3 different experiments (*P<0.05, *vs* WT group).

Supplemental Table 1. Blood parameters of GIT1 WT and KO mice

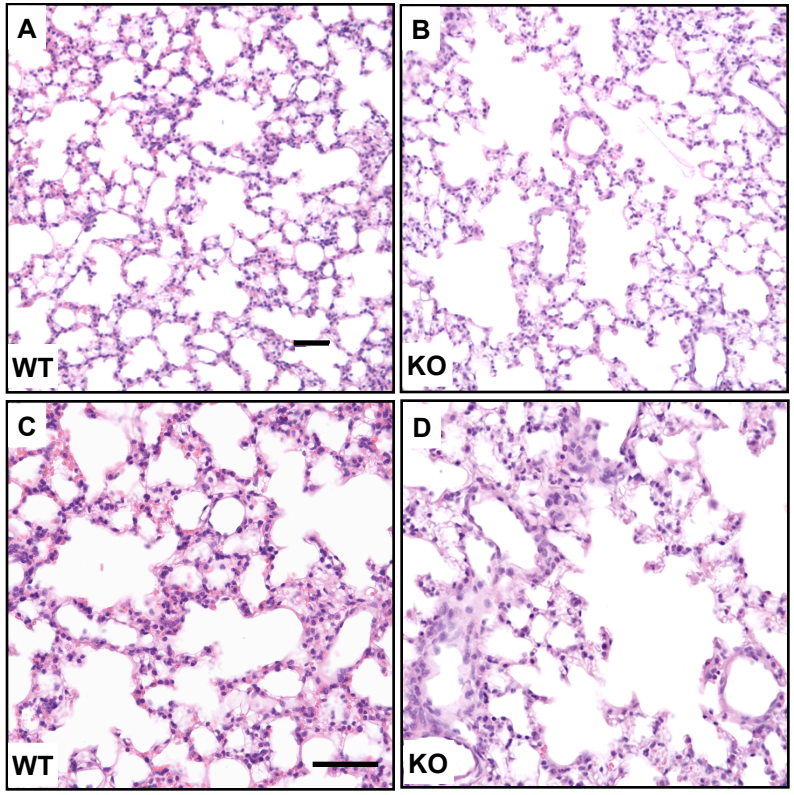
Group / Parameters	GIT1 WT	GIT1 KO
White blood cell (10^3μl)	7.00 ± 0.66	7.50 ± 1.07
Lymphocyte (10^3μl)	5.50 ± 0.46	6.17 ± 1.21
Granulocyte (10^3μl)	0.93 ± 0.20	0.97 ± 0.38
Monocyte (10^3μl)	0.38 ± 0.16	0.30 ± 0.17
Red blood cell (10^6μl)	9.33 ± 0.47	8.89 ± 0.57
Hemoglobin (g/dl)	15.23 ± 0.52	13.73 ± 2.26
Platelets (10^3μl)	557.33 ± 81.37	460.33 ± 76.36

Supplemental Table 2. Hemodynamic parameters

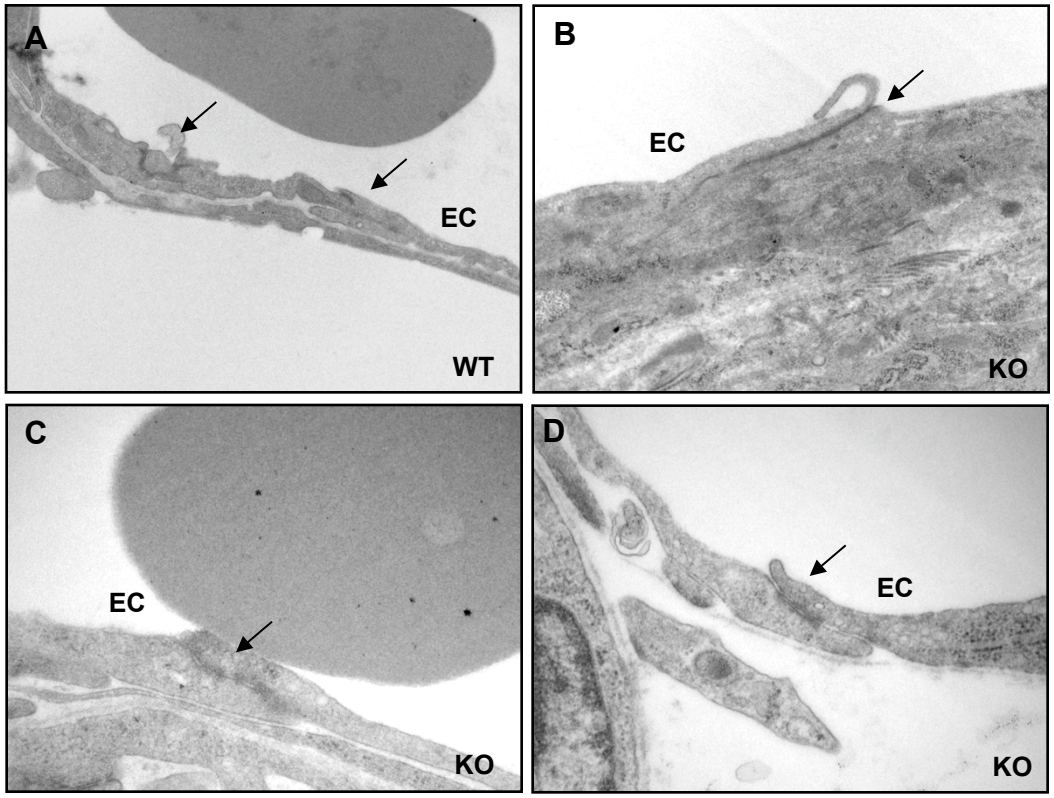
Parameters \ Group	GIT1 WT	GIT1 KO
Heart rate (bmp)	393±27	404±32
Pulmonary artery pressure (mmHg, systolic)	9.11±0.78	11.25±1.17
Right ventricular pressure (mmHg, systolic)	18.08±2.23	20.29±1.01
RV dp/dt_{max}(mmHg/s)	976.51 ± 118.37	932.02±105.43
RV dp/dt_{min} (mmHg/s)	-(974.34±155.27)	-(825.94±108.83)

RV indicates right ventricle. Values are mean ± SE for 6 mice/group

Supplemental Figure 1



Supplemental Figure 2



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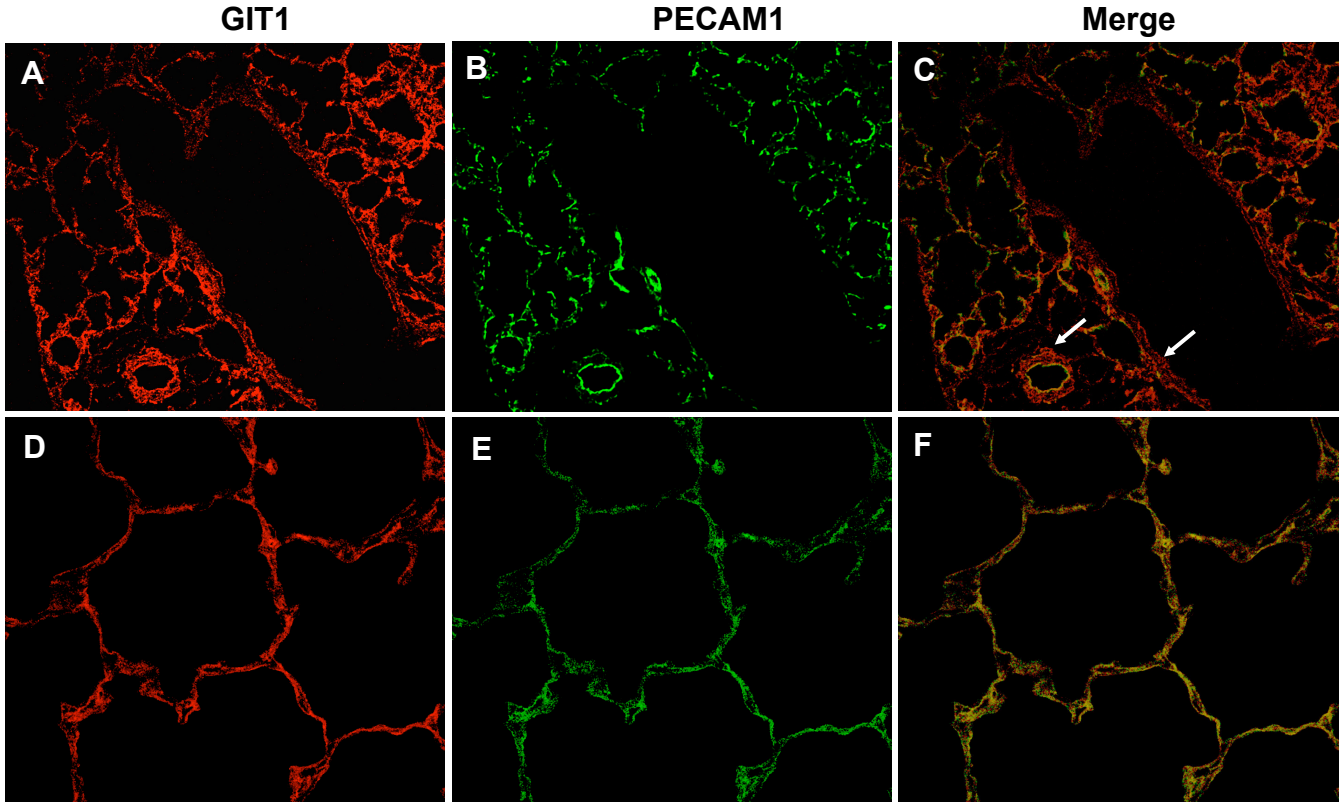

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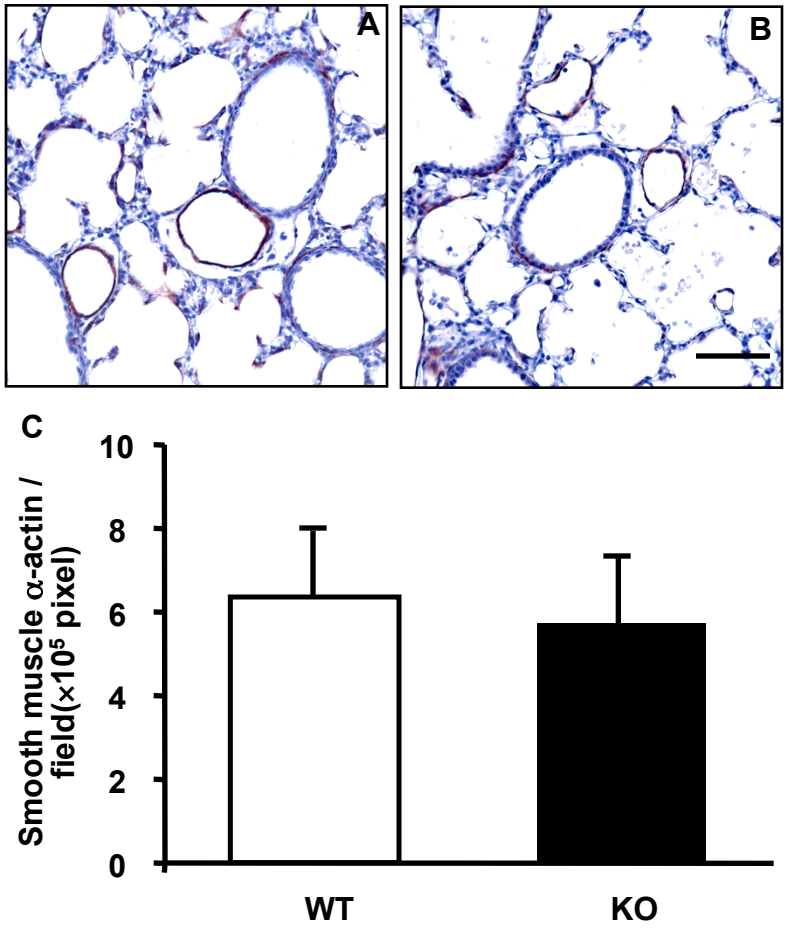
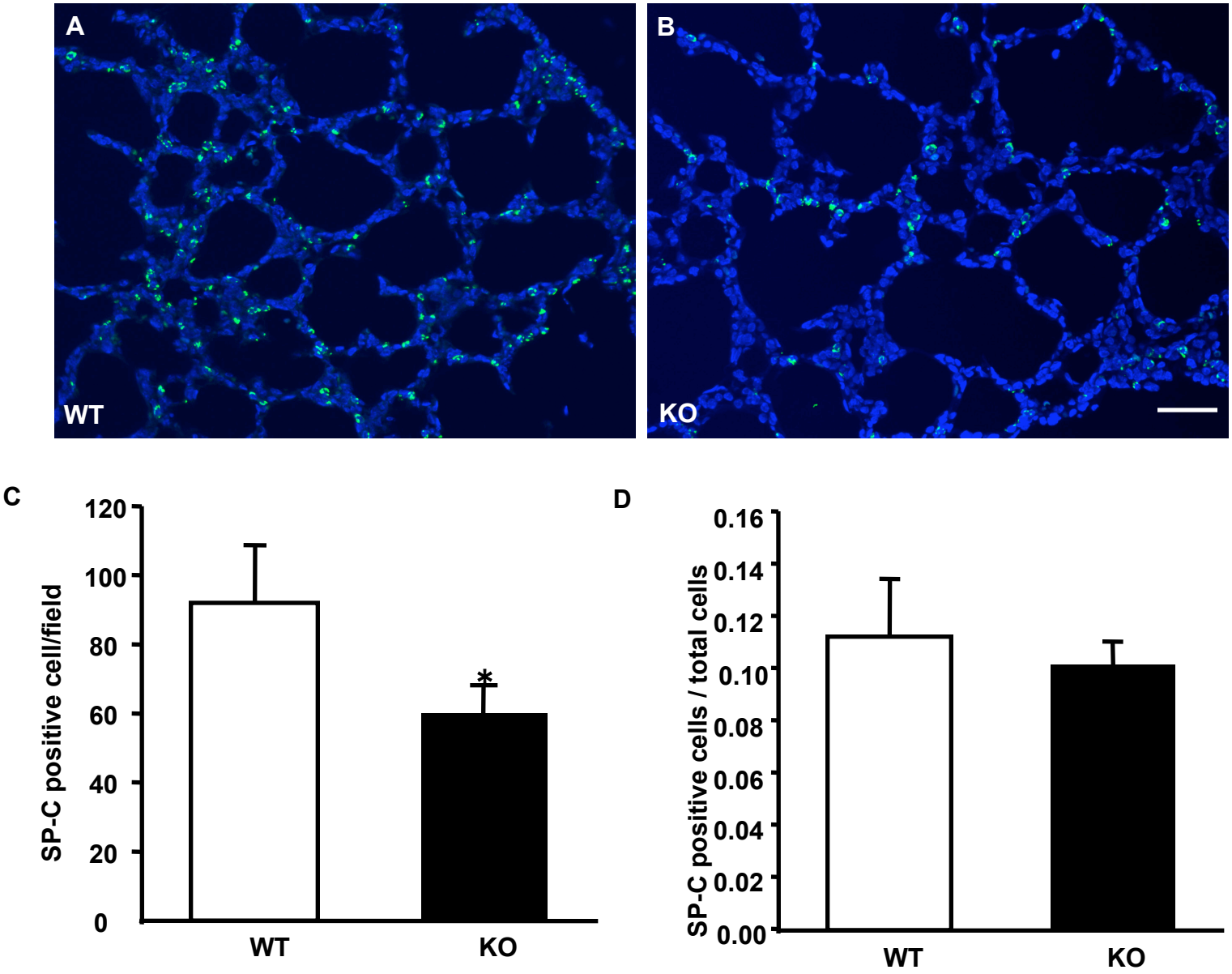
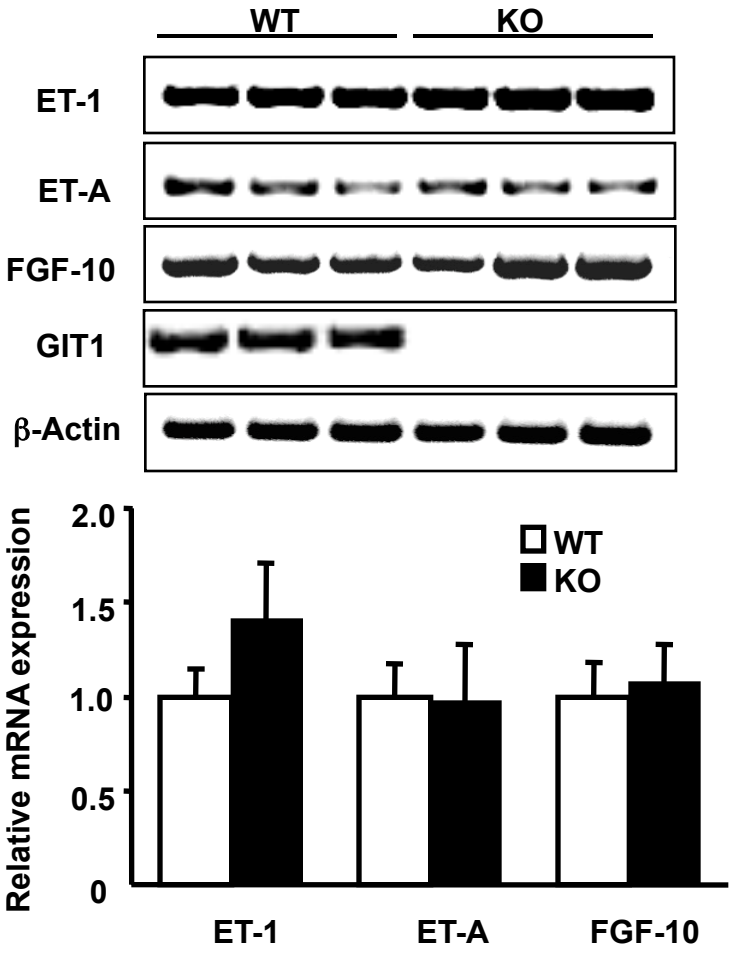


Supplemental Figure 5



Supplemental Figure 6



Supplemental Figure 7