Supporting Information

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SI Methods

Generation of Fbln4 Conditional Knockout Mice. The exon 2 of mouse *Fbln4* was flanked by two loxP sequences inserted in intron 1 and 2. The 5′ loxP sequence was inserted 38 bp upstream the exon 2, by de novo gene synthesis (GeneDesign). The 3′ loxP sequence was inserted 138 bp downstream the exon 2, followed by Flp recognition target (FRT)-flanked neo-cassette. The sites of loxP insertion were determined not to influence the expression of *Fbln4* before excision of the exon 2, by searching sequences less conserved and less likely to contain transcription factor binding site, with Ensembl Genome Browser (http://www.ensembl.org/index.html), University of California Santa Cruz Genome Browser (http://genome.ucsc.edu/), Database of Transcriptional Start Sites (http://dbtss.hgc.jp/), and Transcriptional Factor Search (http://mbs.cbrc.jp/research/db/TFSEARCH.html).

The targeting vector was constructed by modifying bacterial artificial chromosome RP23-411F4 (Invitrogen) using defective prophage λ-Red recombineering system (1–3). The targeting vector was electroporated into C57BL/6 mouse ES cells (DS Pharma Biomedical) with Nucleofector system (Lonza). Positive clones were selected by incubating cells with 200 mM Geneticin (Invitrogen) for 5 days, and homologous recombination was confirmed by Southern blotting using *Fbln4* specific probe indicated in Fig. S1*A*. Successfully recombineered ES cells were injected into blastocysts from ICR strain mice by Unitech, and chimera mice were bred with C57BL/6 mice to generate F1 mice. Genotypes of F1 mice were confirmed by Southern blotting. Neo-cassette flanked by FRT was removed by crossing F1 with CAG-FLPe deleter mice kindly provided by S. Itohara (4).

To generate systemically exon 2-deleted mice (Δ*Ex2*), $Fbln4^{flox/+}$ mice were crossed with *Ayu-1 Cre* knockin mice, which express Cre recombinase in multiple tissues, including germ line (5, 6). Descendant $Fbln4^{\Delta Ex2/+}$ mice without *Ayu-1 Cre* allele were crossed each other to generate $Fbln4^{\Delta Ex2/\Delta Ex2}$ mice.

To generate vascular smooth muscle specific *Fbln4* conditional knockout mice, $Fbln4^{flox/+}$ mice were further crossed with transgenic mice expressing Cre recombinase under *Smooth Muscle Protein 22α* (*Sm22α*) promoter (The Jackson Laboratory) (7, 8). To confirm the expression pattern of Cre recombinase, *Sm22α-Cre* mice were crossed with *Rosa26-lacZ* reporter mice (ROSA26R; The Jackson Laboratory) (9), and subjected to lacZ staining. *Fbln4 null* mice (10) and *Dance null* mice (11) are described elsewhere. All experiments were carried out on C57BL/6 background except for ROSA26R mice that were on mixed background.

Southern Blotting. Genomic DNA samples were purified from tail biopsy specimens and digested with EcoRI. Southern blotting was performed using [^{32}P] dCTP labeled probe indicated in Fig. S1*A*. Primer sequences used to amplify the probe are shown in Table S1.

LacZ Staining. *Sm22α-Cre* mice were bred with ROSA26R mice, and embryos were dissected at 14.5 days postcoitus (dpc). Tissues were fixed with 1% formaldehyde, 0.2% glutaraldehyde, 0.02% Nonidet P-40 in PBS with 2 mM $MgCl_2$, and were embedded in Tissue-Tek OCT compound (Sakura Finetechnical). Sections were then stained as previously described (12, 13), by incubating at room temperature for 140 min in 1 mg/mL X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.02% Nonidet P-40, 0.01% sodium deoxycholate in PBS with 2 mM $MgCl_2$.

Tissue Extraction and Western Blotting. Proteins were extracted from aorta tissues with 8 M urea solution. Neonatal or embryonic tissues were homogenized in PBS, and supernatants were collected. Protein samples of the same amount were subjected to SDS/PAGE (Invitrogen), followed by Western blotting. Anti-FBLN4 polyclonal antibody (109), anti-LOX polyclonal antibody (ab31238; Abcam), or anti-β actin monoclonal antibody (AC-15; Sigma) was used as a primary antibody, horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody (sc2054 and sc2055, respectively; Santa Cruz Biotechnology) was used as a secondary antibody. Signals were detected using Western Lightning Plus Chemiluminescence Reagent (Perkin–Elmer). Rabbit polyclonal anti-FBLN4 antibody (109) was raised by Sigma–Genosys against KLH-conjugated polypeptide CPQGYEPDEQES, which corresponds to amino acids 109–120 of mouse FBLN4 protein.

Histological Examination. For EVG staining, tissues were fixed with 3.7% formaldehyde, and embedded in paraffin blocks. Sections were stained according to standard procedures, and were observed with an Axioskop 2 plus microscope equipped with an Axiocam digital camera (Carl Zeiss).

For transmission electron microscopy (TEM), aorta tissues were fixed with 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer with 0.025% $CaCl_2$. After several washes with 0.05 M cacodylate buffer containing 0.2 M sucrose, specimens were postfixed in 0.1% osmium tetroxide in 0.05 M cacodylate and 0.2 M sucrose buffer, were embedded in either Epon812 (TAAB Laboratories Equipment) or Quetol-651 (Nisshin EM). Thin sections were cut on a Reichert-Nissei ULTRACUT S ultramicrotome (Leica) and treated for 1 h with solution containing 5.7 mg/mL tannic acid (Nacalai Tesque) and 14.3 mg/mL *p*-nitrophenol (Wako Pure Chemical). Sections were counterstained with uranyl acetate followed by lead citrate, and examined with JEM-1400A TEM (JEOL).

Aortography. Mice were anesthetized with pentobarbital (DS Pharma Biomedical), and cannulated in the right carotid arteries. Contrast medium (Iopamidol; Bayer Schering Pharma) was injected in the aorta through the catheter, and the aortograph was observed with an X-ray imaging system OEC 9800 Plus (GE Healthcare) through a left anterior oblique view, to view the entire aortic arch.

Pressure-Diameter Relationship. The aortae were excised and observed as previously described (11). The dimension of aorta that was measured at proximal portion of the descending aorta with a digital image processor CV-2000 (Keyence) and the intraaortic pressure measured with a transducer were simultaneously recorded using a Powerlab data acquisition system (AD Instruments). Numbers of studied mice are indicated in each figure. Each aorta was measured for three times and averaged, and the curves were obtained by plotting mean ± SD for all studied mice. Slope of each curve was calculated from the aortic dimension at 40-mm Hg and 120-mm Hg. Statistical analyses were performed with unpaired *t* test. $P < 0.05$ was considered to be significant.

In Vitro Binding Assay. Full-length cDNA of human *FBLN4* was cloned by reverse transcription PCR from human skin fibroblast

(HSF) RNA. Full-length cDNA of human *LOX* was cloned by PCR using IMAGE cDNA clone 1681885 as a template. Human full-length *DANCE* cDNA is previously described (14).

Expression vector pEF6/V5 (Invitrogen) was modified to attach a preprotrypsin signal sequence followed by a FLAG-tag or a Myc-tag, and a His_6 tag to the N terminus of its inserted protein (named pEF6/FLAG and pEF6/Myc, respectively). FBLN4 FL (nucleotides 276-1526) and LOX FL (nucleotides 439-1629) cDNA were subcloned into both vectors. The FBLN4 ΔN (nucleotides 561-1526), ΔC (nucleotides 276-1169), N (nucleotides 276–560), M (nucleotides 561-1169), C (nucleotides 1170–1526), LOX pro (nucleotides 439–879), LOX mat (nucleotides 880-1629), and DANCE cDNAs were amplified by PCR, and subcloned into pEF6/FLAG. FBLN4 ΔM (nucleotides 276–560, 1170–1526) cDNA fragment was amplified by inverse PCR, followed by self-ligation. Human FBLN4 and LOX cDNA sequences are numbered according to GenBank accession no. NM_016938 and NM_002317, respectively. All constructs were confirmed by sequencing (ABI Prism 3100). Primer sequences used for PCR are provided in Table S3.

HEK293T cells were maintained in DMEM (GIBCO) supplemented with 2 mM glutamine, 100 units/100 μg/mL penicillin/streptomycin, and 10% FBS at 37 °C in 5% CO_2. HEK293T cells were transiently transfected with expression vectors of FLAG-tagged protein or a mock vector using Lipofectamine Reagent (Invitrogen). Expression vectors for Myc-tagged proteins were also independently transfected into 293T cells. Transfected cells were cultured in serum-free DMEM/F12 (Sigma) for 48 h, and then, the cell lysates and the conditioned media were harvested and mixed. After incubation of Myc-tagged proteins with a set of FLAG-tagged proteins, each mixture was subjected to immunoprecipitation with anti-FLAG M2 affinity gel (Sigma) followed by SDS/PAGE and Western blotting as described previously (15).

Immunocytochemistry. HSFs were cultured on microscope cover glasses (Fisherbrand), in DMEM/F12 (Sigma) supplemented with 2 mM glutamine, 100 units/100 μg/mL penicillin/streptomycin, and 10% FBS at 37 °C in 5% CO_2, and were stained as previously described (15). The primary antibodies used were anti-LOX polyclonal (NB-100-2530; Novus Biologicals), anti-elastin polyclonal (PR533; EPC), and anti-FBLN4 monoclonal (9C10) antibodies. The secondary antibodies used were AlexaFluor 488-conjugated anti-rabbit and AlexaFluor 546-conjugated anti-mouse IgG antibodies (Invitrogen), followed by nuclear staining with Hoechst 33258 (Dojindo). Antibodies were diluted in Can Get Signal immunostain solution (TOYOBO) according to the manufacturer's instruction. Stained cells were examined using a Nikon Digital Eclipse C1si Laser Confocal system. Anti-FBLN4 monoclonal antibody (9C10) was raised against recombinant full-length human FBLN4 by TransGenic.

Solid-Phase Binding Assay. HEK293T cells were transfected with pEF6/Myc-human FBLN4 with Lipofectamine Reagent (Invitrogen), according to the manufacturer's protocol. Stably transfected cells were selected with Blasticidin (Invitrogen), and recombinant FBLN4 was purified from the serum-free conditioned medium of stable lines with TALON His-Tag Purification resins (Takara). The purity of the protein was confirmed by Coomassie blue staining of a SDS/PAGE gel, and the protein concentration was determined with Coomassie Plus Reagent (Pierce).

Solid-phase binding assay using purified tropoelastin was performed as described previously with some modifications (10). As soluble ligands, conditioned media of 293T cells transfected with pEF6/FLAG-LOX FL were used. Cells were transfected with the plasmid as described above, and were cultured in serum-free DMEM/F12 (Sigma) for 48 h. The conditioned medium was harvested and was concentrated 10-fold by ultrafiltration in Centricon Ultracel YM-10 (Millipore). The concentrated sample was serially diluted with Tris-buffered saline at a final concentration of 2% skim milk with 2 mM $CaCl_2$, with or without 40 μg/mL of recombinant FBLN4. These mixtures were used as ligands for the assay. Anti-FLAG M2 antibody (Sigma) and horseradish peroxidase-conjugated anti-mouse IgG antibody (Santa Cruz) was used as a primary antibody and a secondary antibody, respectively. A color reaction assay was performed with Substrate Reagent Pack (R&D), followed by optic density measurement at 450 nm. Data are presented as means ± SD of three independent experiments.

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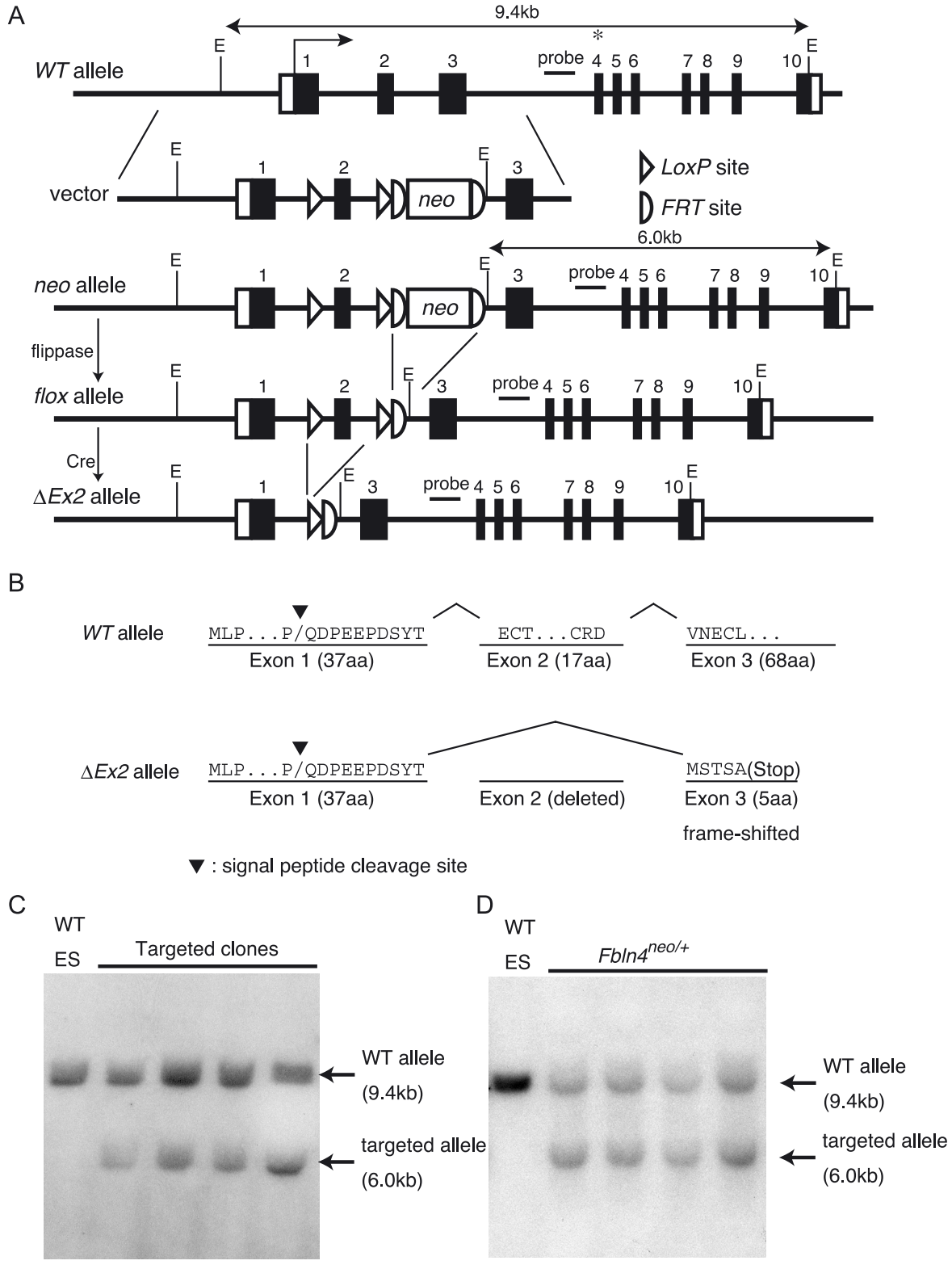

Fig. S1. Generation of *Fbln4* conditional knockout allele. (*A*) Schematic representation of the strategy to target the *Fbln4* allele. Asterisk indicates the exon where the *neo* cassette is inserted in the previously reported *Fbln4 null* mice. E, EcoRI site; *FRT*, flippase recognition target; *neo*, neomycin resistant cassette. (*B*) Predicted peptide translated after excision of exon 2. Deletion of exon 2 leads to frame-shift, and translation will be terminated at the 5th amino acid of exon 3, producing a peptide of 15-aa length after cleavage of the signal peptide. (*C*) Southern blotting of successfully targeted ES clones. (*D*) Southern blotting of F1 mice tail DNA. Genomic DNA of wild-type ES cell was used as a control.

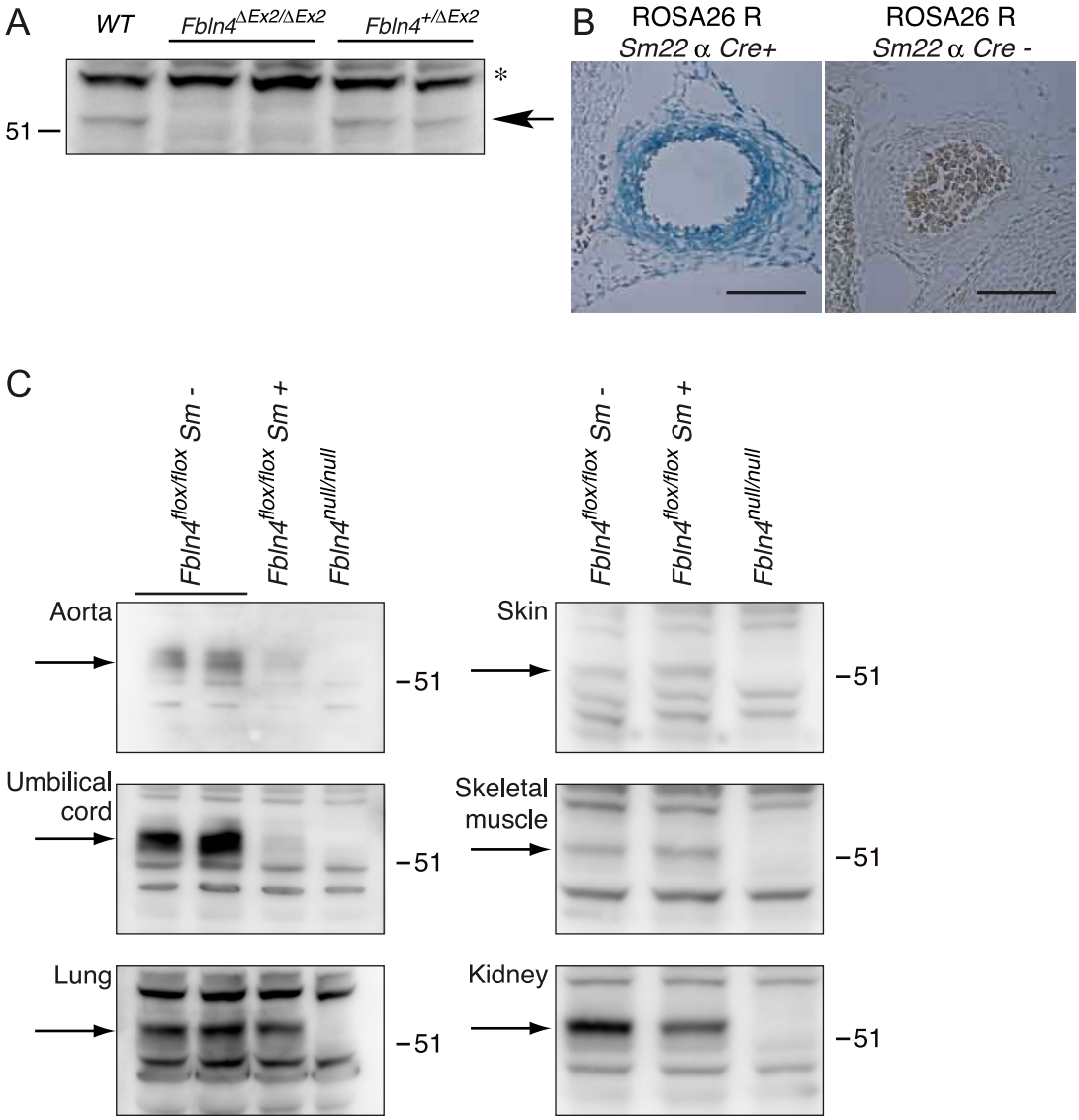

Fig. S2. Expression of FBLN4 in conditional knockout mice. (*A*) Loss of FBLN4 expression in neonatal lungs of *Fbln4*$^{\Delta Ex2/\Delta Ex2}$ mice. Lung extracts were analyzed by Western blotting using anti-FBLN4 antibody. Asterisk indicates nonspecific bands that serve as a loading control. (*B*) Efficiency of recombination of *Sm22*α *Cre* transgenic mice in aorta at 14.5 dpc. Recombination by Cre recombinase was made visible by LacZ staining of the embryos from the cross of *Sm22*α *Cre* mice and *ROSA26R* indicator mice. (Scale bar, 100 μm.) (*C*) Western blotting of extracts from various tissues of *Fbln4*$^{flox/flox}$ *Sm*+ embryos at 16.5 dpc shows deletion of the expression of FBLN4 in the aorta or umbilicus. However, FBLN4 was expressed in the lung, skin, skeletal muscle, and kidney at levels comparable with wild type.

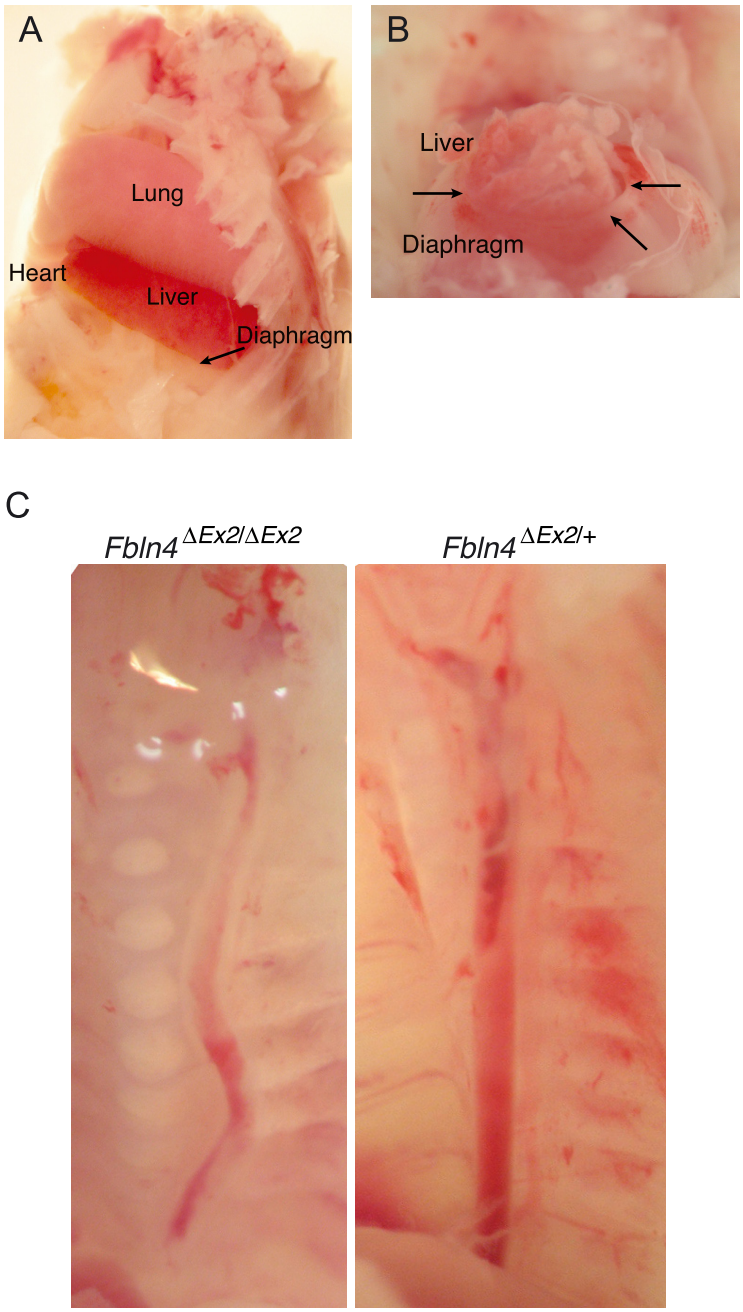

Fig. S3. Diaphragmatic hernia and tortuous aorta in *Fbln4*$^{\Delta Ex2/\Delta Ex2}$ neonate. (*A*) Dissection of *Fbln4*$^{\Delta Ex2/\Delta Ex2}$ neonate, left anterior oblique view after removal of anterior chest wall. A part of liver is protruding into the thoracic cavity. (*B*) Diaphragmatic hernia of *Fbln4*$^{\Delta Ex2/\Delta Ex2}$ neonate. A large defect (arrows) is present at the middle of the diaphragm. The image was taken after removal of a part of the liver bulged into the thoracic cavity. (*C*) The aorta of *Fbln4*$^{\Delta Ex2/\Delta Ex2}$ mice is tortuous compared with *Fbln4*$^{\Delta Ex2/+}$ mice.

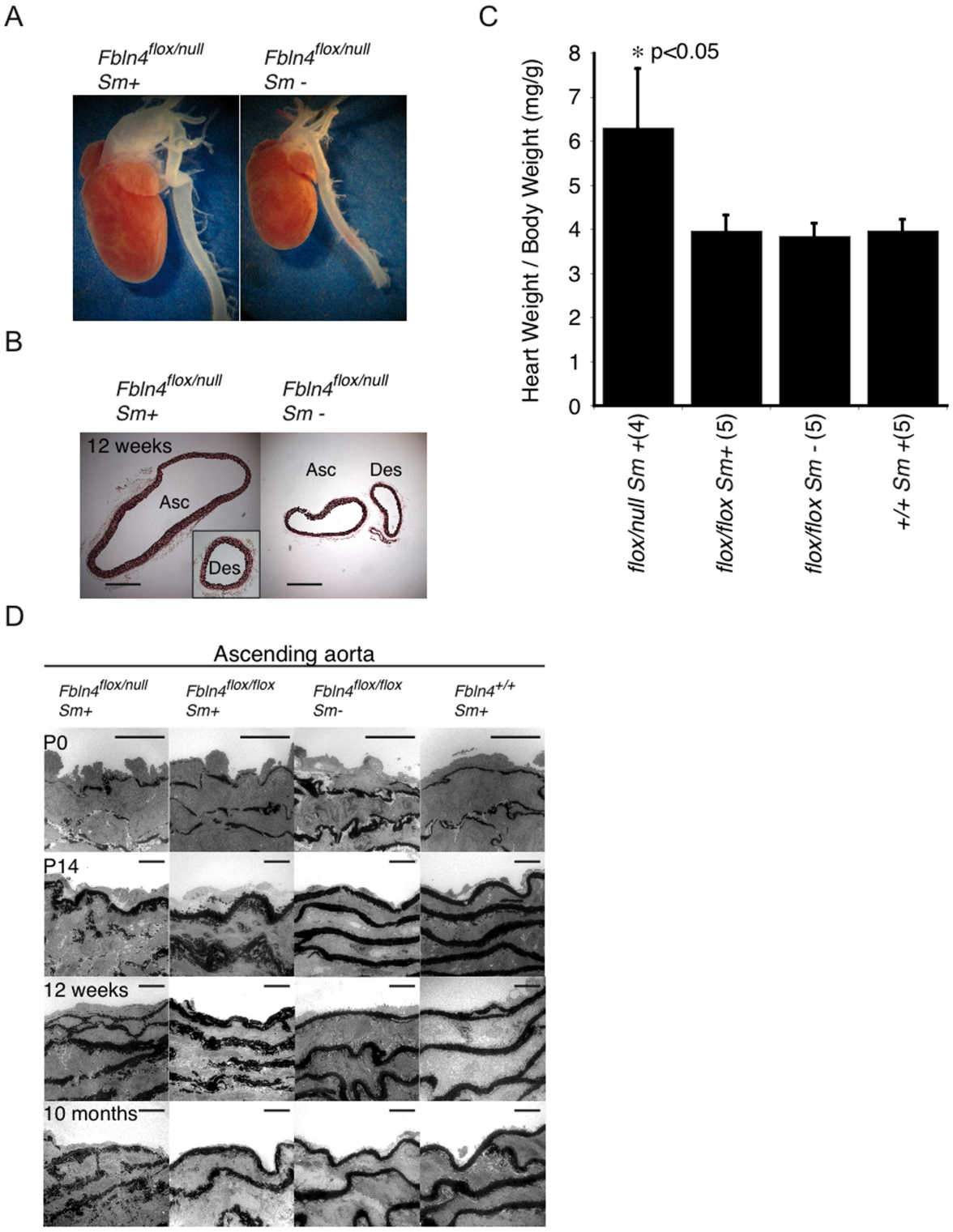

Fig. S4. Phenotype of *Fbln4* conditional knockout mice. (*A*) Macroscopic appearance of the heart and aorta of *Fbln4*$^{flox/null}$ *Sm*+ and *Sm*− mice. Aneurysm of the ascending aorta and LV hypertrophy is evident in *Fbln4*$^{flox/null}$ *Sm*+ mice. (*B*) Low magnification view of EVG staining of the aortae at 12 weeks. The ascending aorta of *Fbln4*$^{flox/null}$ *Sm*+ mice is markedly dilated. Asc, ascending aorta; Des, descending aorta. (Scale bar, 500 μm.) (*C*) Heart weight of male mice was compared. Numbers of studied mice are indicated in parentheses. Hypertrophy is evident in *Fbln4*$^{flox/null}$ *Sm*+ mice. (*D*) TEM of mice aortae at postnatal day (P)0, P14, 12 weeks, and 10 months. Elastic laminae of *Fbln4*$^{flox/null}$ *Sm*+ mice are already affected at P0, whereas those of *Fbln4*$^{flox/flox}$ *Sm*+ mice are not. Abnormal disrupted elastic laminae are observed at P14 in *Fbln4*$^{flox/flox}$ *Sm*+ mice. Note that elastic laminae are thicker and spongy in conditional knockout mice, and that there are aberrant deposits in interlaminar spaces. Internal elastic lamina is lost in the ascending arteries of *Fbln4*$^{flox/null}$ *Sm*+ mice. (Scale bar, 10 μm.)

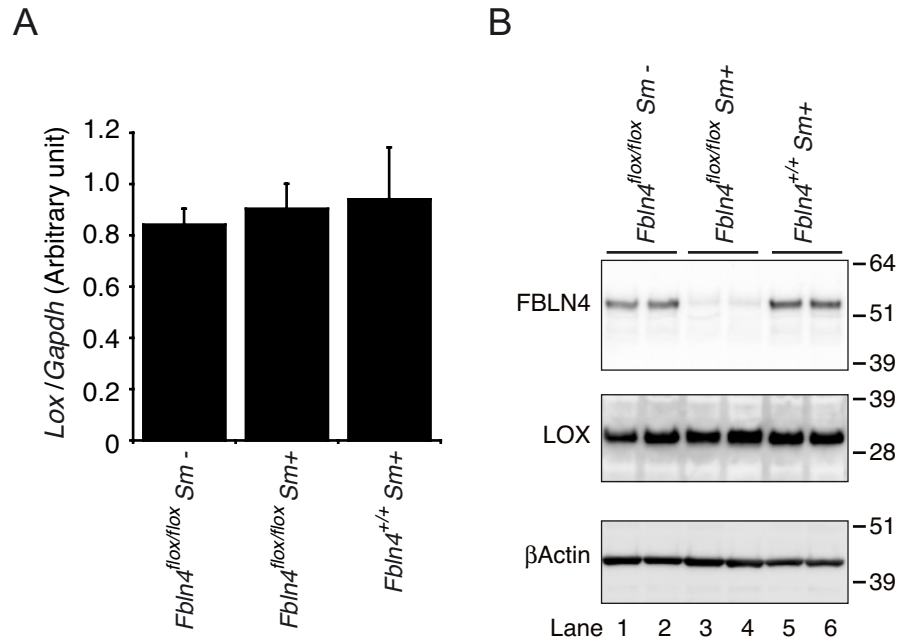

Fig. S5. Expression of LOX in *Fbln4* conditional knockout mice. (*A*) Quantitative PCR analysis of neonatal aorta shows that the expression of *Lox* is not affected by the disruption of *Fbln4*. The expression level of *Lox* was normalized by that of *Gapdh*, and relative expression level of *Lox* in a wild-type mouse was defined as 1.0. Data are represented as means ± SD of three independent experiments each performed in duplicate. (*B*) FBLN4 is undetectable in $Fbln4^{flox/flox}$ *Sm*+ mice aortae by Western blotting (*Top*, lanes 3 and 4), whereas the amount of LOX is similar to that of control mice (*Middle*, compare lanes 3 and 4 with lanes 1, 2, 5, and 6). The expression level of β actin was used as a loading control (*Bottom*).

Table S1. Primer sequences to generate probe for Southern blotting

Sense	Antisense
5′-AACCGTAAGGGCTTTGACTGAGGAC-3′	5′-CTTGTCCATTTACACCCCTCAGCTG-3′

Table S2. Primer sequences for quantitative PCR

	Sense	Antisense
Fbln4	5′-ATGGCTATGAGTGGGATGCAGACAGCCAGC-3′	5′-TGGCAATAGCGGTAACGACACTCATCTATG-3′
Gapdh	5′-CCATCACCATCTTCCAGGAG-3′	5′-CACACCCATCACAAACATGG-3′
Lox	5′-GCCCTCGGTACTCCTGGGAGTGGCACAG-3′	5′-AGACAGAAGCTTGCTTTGTGGCCTTCAG-3′

Gapdh, glyceraldehyde-3-phosphate dehydrogenase; *Lox, lysyl oxidase*

Table S3. Primer sequences for plasmid construction

	Sense	Antisense
Fbln4 cDNA	5′-GACACTAGTATGCTCCCCTGCGCCTCCTGCCTACCC-3′	5′-GACGGATCCTCAGAAGGTGTAGGCCCCTACAAAGAC-3′
Fbln4 FL	5′-GCGTCTAGACAGGATTCTGAAGAGCCCGACAGCTAC-3′	5′-GACGGATCCTCAGAAGGTGTAGGCCCCTACAAAGAC-3′
Fbln4 ΔN	5′-GGTCTAGAGATGTGGACGAGTGTGCCCAGGCCCTG-3′	5′-GACGGATCCTCAGAAGGTGTAGGCCCCTACAAAGAC-3′
Fbln4 ΔM	5′-CGCTGTCTCTGCCCGGCCTCCAACC-3′	5′-CACACAGCTGTCCTGATCGTCGGGCTC-3′
Fbln4 ΔC	5′-GCGTCTAGACAGGATTCTGAAGAGCCCGACAGCTAC-3′	5′-CTGGATCCTAGTTCTCAGAGACCTGGATGTAGGGCTC-3′
Fbln4 N	5′-GCGTCTAGACAGGATTCTGAAGAGCCCGACAGCTAC-3′	5′-CTGGATCCTACACACAGCTGTCCTGATCGTCGGGCTC-3′
Fbln4 M	5′-GGTCTAGAGATGTGGACGAGTGTGCCCAGGCCCTG-3′	5′-CTGGATCCTAGTTCTCAGAGACCTGGATGTAGGGCTC-3′
Fbln4 C	5′-GGTCTAGACGCTGTCTCTGCCCGGCCTCCAAC-3′	5′-GACGGATCCTCAGAAGGTGTAGGCCCCTACAAAGAC-3′
Lox FL	5′-CTACTAGTGCCCCTCCCGCCGCCGGCCAACAGCAG-3′	5′-GCGGATCCTAATACGGTGAAATTGTGCAGCCTG-3′
Lox pro	5′-CTACTAGTGCCCCTCCCGCCGCCGGCCAACAGCAG-3′	5′-CTGGATCCTAGCCCACCATGCCGTCCACGCGGCTGG-3′
Lox mat	5′-CGACTAGTGACGACCCTTACAACCCCTACAAGTAC-3′	5′-GCGGATCCTAATACGGTGAAATTGTGCAGCCTG-3′

Fbln, *fibulin*; *Lox*, *lysyl oxidase*.