Supporting Information

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SI Materials and Methods

Aorta Extensibility Measurements. The diameters of the extirpated aortae and the intraaortic pressure were measured as previously described (1). The slope was calculated from the aortic dimension at 40 and 120 mm Hg.

Electron Microscopy. For transmission electron microscopy, ascending aortae were fixed in 2% glutaraldehyde and 1 mM $CaCl_2$ in 0.05 M sodium cacodylate buffer at 4 °C overnight. Samples were postfixed in 1% osmium tetroxide and processed for Epon embedding. Thin sections (95 nm) were stained with tannic acid and *p*-nitrophenol followed by counterstaining with uranyl acetate and lead citrate. Electron micrographs were taken with JEM-1400A (JEOL).

Cell Culture for Elastic Fiber Development. To develop elastic fibers, confluent HDFs at passage 8 were seeded on noncoated glass coverslips (Fisher) at 8×10^4/well in a 24-well plate, and on the next day the medium was changed to 1 mL/well of DMEM/F12 (Invitrogen) supplemented with 2 mM glutamine, 100 units/100 mg·mL^{-1} penicillin/streptomycin, and 10% FBS. The cells were kept at 37 °C in 5% CO_2 for 12–14 d without changing the medium.

Antibodies. All secondary antibodies conjugated with Alexa 488 or 546 for immunofluorescence, with peroxidase for immunohistochemistry, were purchased from Invitrogen. Paraffin sections of fixed tissues were subjected to immunofluorescence staining (Fig. 1). Immunohistochemical staining of paraffin sections were performed by the avidin-biotin-peroxidase complex method (ABC-Elite; Vector Laboratories) (Fig. 5). Immunofluorescence staining of HDFs (Figs. 3 and 4, Figs. S6, S7, S9, and S10) was performed as previously described (2), and visualized using BZ-9000 microscope (Keyence) with GFP-BP, TRITC, and DAPI-BP filter cubes, CFI Plan Apo 40× NA 0.95 lens (Nikon), and a built-in 2/3 inch, 1.5 megapixel, 12-bit, monochrome cooled CCD camera (Keyence). Image files were taken in TIFF format using the operating software, merged, linearly contrast stretched (with the same setting in each set of experiments) using Photoshop CS5 (Adobe), and imported into Illustrator CS5 (Adobe) for assembly.

Plasmid Construction. The following fragments of *LTBP4S, LTBP4L*, and their fragment cDNAs were amplified by PCR, and subcloned into pEF6/ssFLAG (preprotrypsin signal sequence, an FLAG tag and a 6× His tag at the N terminus), pEF6/FLAG (an FLAG tag and a 6× His tag at the C terminus) or pEF6/Myc (a Myc tag and a 6× His tag at the C terminus) (3): LTBP-4L-A (exons 2–10), LTBP-4S-A (exons 6–10), LTBP-4-B (exons 11–13), LTBP-4-C (exons 14–20), LTBP-4-D (exons 21–27), LTBP-4-E (exons 28–31), LTBP-4-F (exons 32–35), LTBP-4L-G (exon 2–5), LTBP-4S-G (exon 6), LTBP-4-H (exon 7), and LTBP-4-I (exons 8–10). LTBP-4L-G-Ig, LTBP-4S-G-Ig, LTBP-4-H-Ig, and LTBP-4-I-Ig were C-terminally fused with the Fc region of human IgG. Human full-length *FBLN5* cDNA was cloned as previously described (4). The expression vectors of full length-, ΔN1-, ΔN2-, ΔM-, and ΔC-fibulin-5 were prepared as previously described (2). The cDNAs of *N*-, M-, C-, and NM-fibulin-5 fragments were amplified by PCR, and subcloned into pEF6/ssFLAG. These fibulin-5 truncation mutants were C-terminally fused with the Fc region of mouse IgG. The cDNA for eLAP corresponding to 1–278 aa of human TGFβ1 precursor was PCR-amplified from HDF cDNA with additional stop codon at the 3′ end, and subcloned to pLenti 6.3 vector (Invitrogen).

Protein Purification. Recombinant LTBP-4S (rLTBP-4S) with an FLAG tag and a 6x-His tag was purified using TALON affinity resin (Takara) from serum-free conditioned medium of 293T cells stably transfected with pEF6/FLAG-LTBP-4S or pEF6/ssFLAG-LTBP-4S. Purification of recombinant fibulin-5 was described previously (2). To prepare rLTBP-4S + eLAP, tagged LTBP-4S-expressing 293T cells were infected with lentiviral vectors expressing eLAP without a tag, followed by purification with TALON affinity resin from serum-free conditioned medium. Recombinant tropoelastin was expressed in bacteria and purified as previously described (2). The purity of each protein was confirmed by Coomassie blue staining of an SDS/PAGE gel. The protein concentration was quantified from scanned gel images with ImageJ software using BSA as a standard.

RNAi. The oligonucleotides sequences are as follows:
Control sense 5′-AAACCCGGUAUGUCACUCUGUCAGC-3′,
antisense 5′-GCUGACAGAGUGACAUACCGGGUUU-3′;
LTBP4-2 sense 5′-ACCGAGACAGCUGAGUACCAGUCAU-3′,
antisense 5′-AUGACUGGUACUCAGCUGUCUCGGU-3′;
LTBP4-3 sense 5′-UGUGCAAGAGUGGCGUGUGUGUGAA-3′,
antisense 5′-UUCACACACACGCCACUCUUGCACA-3′;
FBLN5-1 sense 5′-CCAGUCAGGACAGUGUUUAGAUAUU-3′,
antisense 5′-AAUAUCGAAACACUGUCCUGACUGG-3′;
FBLN5-2 sense 5′-CCCACCACUCUCAGCUCCAAACUAU-3′,
antisense 5′-AUAGUUUGGAGCUGAGAGUGGUGGG-3′;
FBLN5-3 sense 5′-GGCCUCUUAUAUGCCGCUUUGGAUA-3′,
antisense 5′-UAUCCAAAGCGGCAUAUAAGAGGCC-3′.

qPCR. Total RNA was extracted using RNeasy Plus Mini Kit (QIAGEN) and transcribed to cDNA with random hexamers using SuperScript III First-Strand Synthesis System (Invitrogen). For qPCR, the reaction was performed with QuantiTect SYBR Green PCR Kit (QIAGEN), and the products were analyzed with Rotor-Gene Q (QIAGEN). The following primer sequences were used:
GAPDH forward 5′-AGGTGAAGGTCGGAGTCAACG-3′,
reverse 5′-GATGACAAGCTTCCCGTTCTCAG-3′;
LTBP4 forward 5′-GAGACAGCTGAGTACCAGTCATT,
reverse 5′-CTGTGTGTGGTAGTAGTAGCCGT-3′;
FBLN4 forward 5′-TTGATGTGAACGAGTGTGACATGG,
reverse 5′-CAGAGGTAGCTGGAGTAGCTACAC;
FBLN5 forward 5′-ATACTCACTGTTACCATTCTGGCT-3′,
reverse 5′-GGTTAACACACATCATGTCTCCTC-3′;
ELN forward 5′-AGTTGGTGGCTTAGGAGTGTCT-3′,
reverse 5′-TTAACTCCTGCTCCAGTGGGAACT-3′;
LOX forward 5′-GAGTCCTGGCTGTTATGATACCTA-3′,
reverse 5′-GTATAGTCAGATTCAGGAACCAGG-3′;
LOXL1 forward 5′-TCAAGCGCTATGCATGCACCTCTCATA-3′,
reverse 5′-GATGTCCGCATTGTAGGTGTCATAGCA-3′;
FBN1 forward 5′-CATTAAGTGCACTGATCTGGAC-3′,
reverse 5′-ATTCAGGTTCTCAGAGCACTCAT-3′.

Quantitative Measurement of Insoluble Elastin. The 8×10^5 HDFs transfected with each siRNA were plated on 60-mm dishes. Three days after plating, 20 mCi of [^{3}H]valine (American Radiolabeled Chemicals) was added to each dish, together with recombinant

LTBP-4S protein. The cultures were incubated at 37 °C in 5% CO_2 for 10 d. The cells were harvested in 0.1 M acetic acid on ice. After centrifugation, the pellets were boiled in 0.1 N NaOH for 15 min followed by centrifugation, and the pellets were again boiled in 0.1 N NaOH for 1 h. Subsequently, the NaOH-insoluble pellets were boiled with 5.7 N HCl for 1 h, mixed with scintillation fluid, and measured for radioactivity with Tri-Carb 2100TR (Packard Bioscience).

TGF-β Activity Assay. Mink lung epithelial cells stably transfected with a luciferase gene driven by TGF-β–responsive plasminogen activator inhibitor-1 promoter sequences (TMLECs) were allowed to attach for 3 h in a 96-well plate. DMEM/0.1% BSA containing TGF-β standards or recombinant proteins were heat-treated and added to TMLECs. After 16 h, TGF-β activities were assessed by measuring luciferase activity in cell lysates. Luciferase activities were measured using ONE-Glo Luciferase Assay System (Promega).

1. Horiguchi M, et al. (2009) Fibulin-4 conducts proper elastogenesis via interaction with cross-linking enzyme lysyl oxidase. *Proc Natl Acad Sci USA* 106(45):19029–19034.
2. Hirai M, et al. (2007) Fibulin-5/DANCE has an elastogenic organizer activity that is abrogated by proteolytic cleavage in vivo. *J Cell Biol* 176:1061–1071.
3. Hirai M, et al. (2007) Latent TGF-beta-binding protein 2 binds to DANCE/fibulin-5 and regulates elastic fiber assembly. *EMBO J* 26(14):3283–3295.
4. Yanagisawa H, et al. (2002) Fibulin-5 is an elastin-binding protein essential for elastic fibre development in vivo. *Nature* 415(6868):168–171.
5. Liu X, et al. (2004) Elastic fiber homeostasis requires lysyl oxidase-like 1 protein. *Nat Genet* 36(2):178–182.

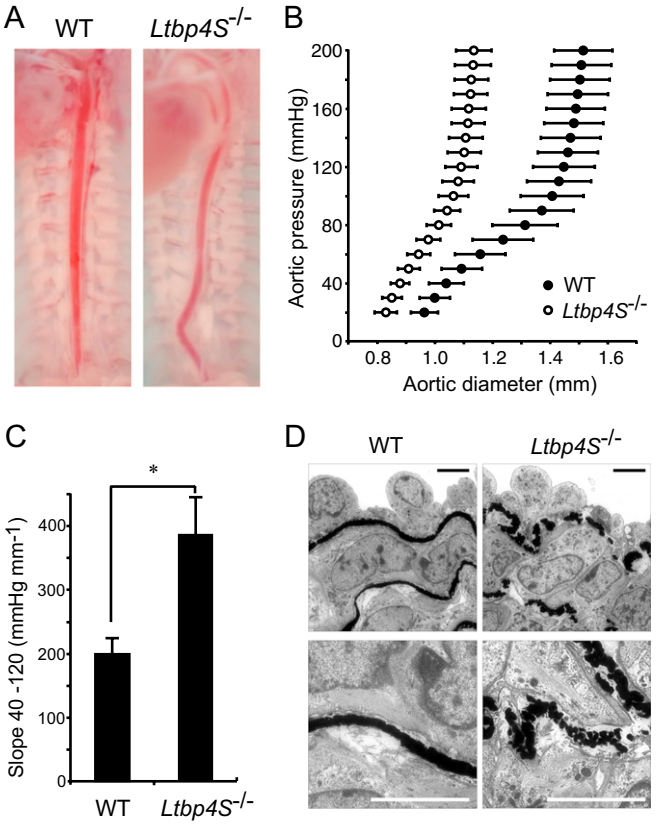

Fig. S1. Vascular phenotypes of *Ltbp4S*$^{-/-}$ mice. (*A*) Aortae of WT and *Ltbp4S*$^{-/-}$ mice at P5. Note tortuosity of the aorta of *Ltbp4S*$^{-/-}$ mice. (*B*) Pressure–diameter relationship of aorta explants of 4-wk-old mice ($n = 6$ for each genotype). Aortic diameter was measured while continuously changing the intraluminal pressure. (*C*) Slopes of pressure–diameter curves between 40 and 120 mm Hg. Aortae of *Ltbp4S*$^{-/-}$ mice were significantly less extensible than control aortae in the range of physiological pressure (*$P = 0.00003$, Student's t test, $n = 6$ for each genotype). Error bars represent SD. (*D*) Transmission electron micrographs of ascending aortae from WT and *Ltbp4S*$^{-/-}$ mice at P2. Note disorganized elastic lamellae stained in black by tannic acid, in *Ltbp4S*$^{-/-}$. (Scale bars, 5 μm.)

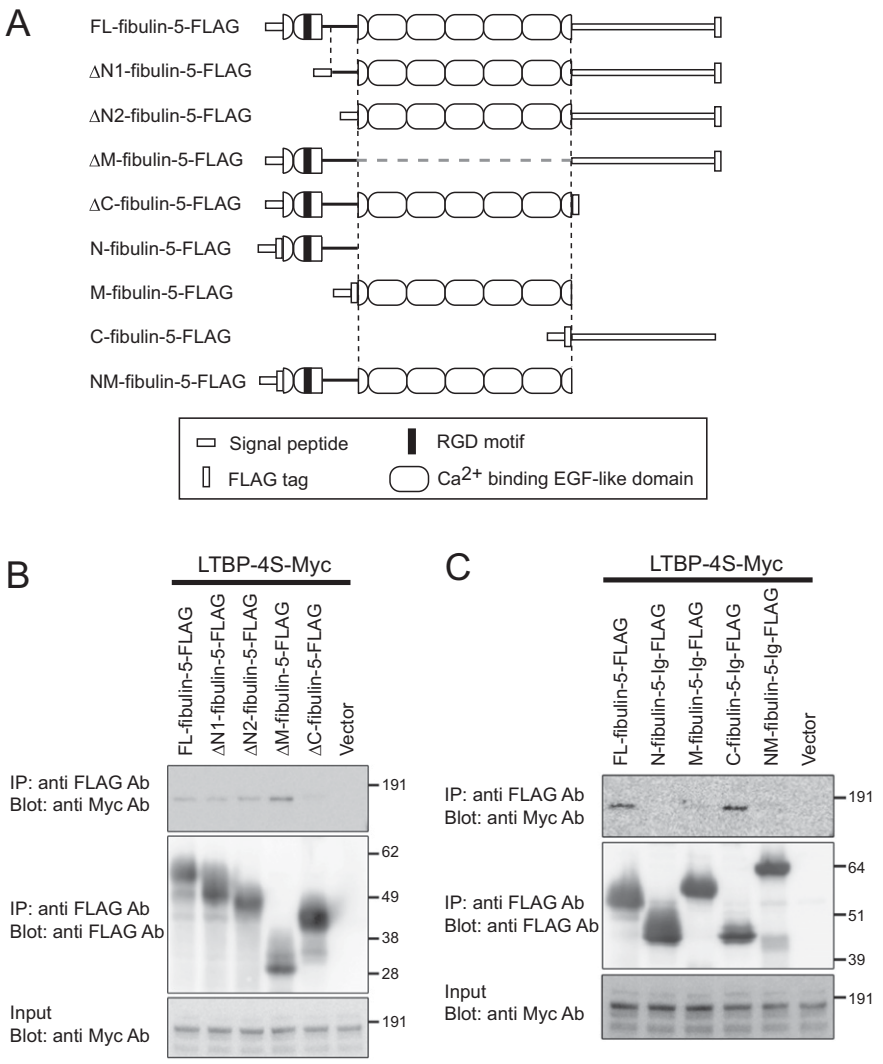

Fig. S2. In vitro binding assay, showing interaction of LTBP-4 with fibulin-5. The expression vectors were independently transfected into 293T cells. Transfected cells were cultured in serum-free medium for 48 h, and the cell lysates and the conditioned media were harvested. Mixtures of the media and cell lysates were incubated with each other, and these reactants were subjected to immunoprecipitation with anti-FLAG antibody. The immunoprecipitants were separated by SDS/PAGE, and analyzed by Western blotting. (*A*) Domain structure of the full-length fibulin-5 and the fibulin-5 deletion/truncation mutants. The deletion mutants were expressed as C-terminal FLAG-tagged proteins. The truncation mutants were expressed as N-terminal FLAG-tagged proteins flanked by preprotrypsin signal sequence and C-terminally fused to the Fc region of murine IgG. (*B*) Interaction of LTBP-4S with full-length fibulin-5 and with the deletion mutants lacking the N-terminal domain or the central calcium binding EGF-like repeat domains, but not with the deletion mutant lacking the C-terminal domain. (*C*) Interaction of LTBP-4S with the fibulin-5 truncation mutant containing the C-terminal domain, but not with other domains.

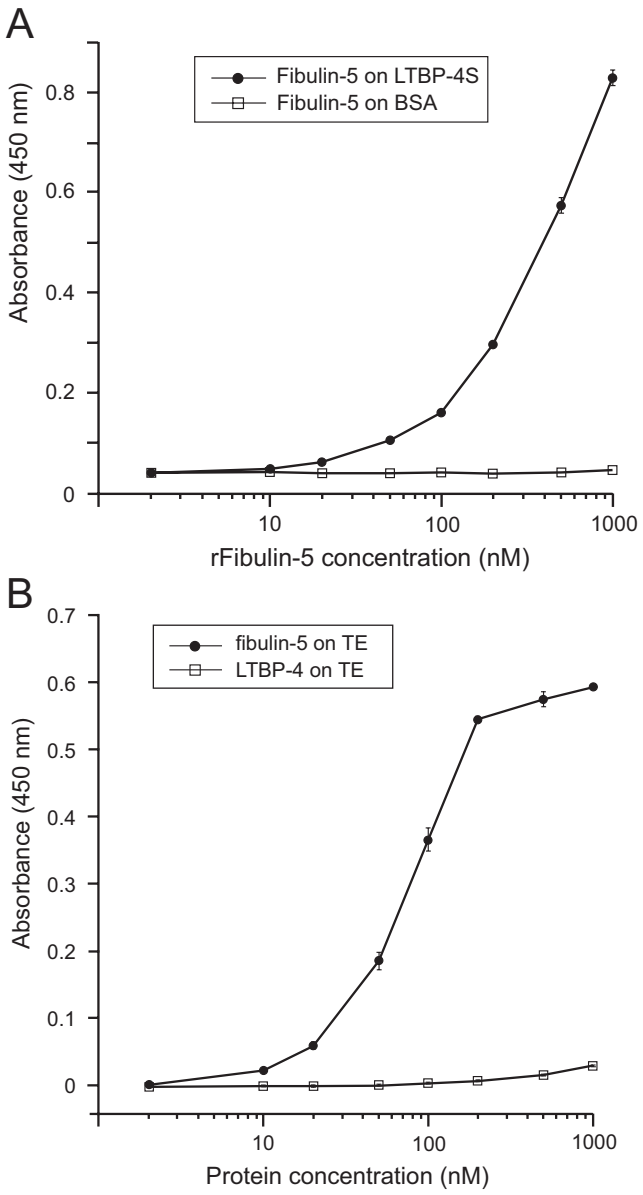

Fig. S3. Solid-phase binding assays. (*A*) Fibulin-5 directly interacts with LTBP-4. Solid-phase binding assays on recombinant LTBP-4S or BSA were performed using variable concentrations of recombinant FLAG-tagged fibulin-5 as soluble ligands. After washing, FLAG-tagged proteins bound to LTBP-4S or BSA were detected using anti-FLAG antibody. (*B*) Fibulin-5 strongly interacts with tropoelastin, whereas LTBP-4 does not directly interact with tropoelastin. Solid-phase binding assays on recombinant tropoelastin or BSA were performed using variable concentrations of recombinant FLAG-tagged LTBP-4S or FLAG-tagged fibulin-5 as soluble ligands. After washing, FLAG-tagged proteins bound to tropoelastin or BSA were detected using anti-FLAG antibody. Signals detected for binding on BSA were subtracted as nonspecific background from signals detected for binding on tropoelastin. All measurements were performed in triplicate, and values shown are means ± SD.

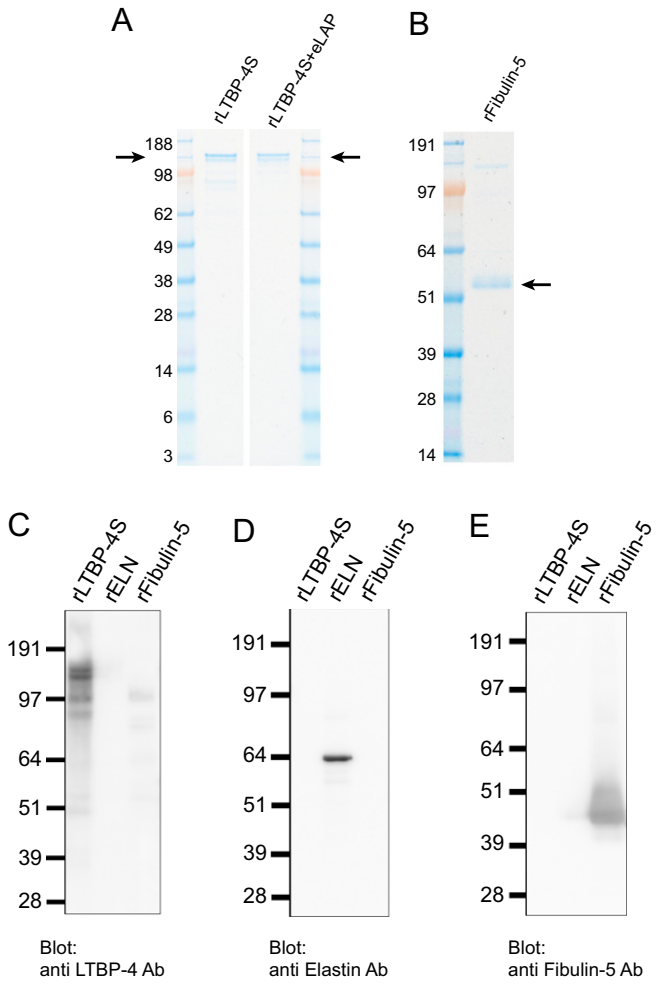

Fig. S4. Purity of the recombinant proteins and specificity of the antibodies used in this study. 6× His-tagged rLTBP-4S, rLTBP-4S + eLAP (*A*) and rFibulin-5 (*B*) were purified by chelating chromatography from culture media of 293T clones stably overexpressing the respective proteins. A Coomassie blue–stained SDS/PAGE gel at a reduced condition of a representative preparation is shown. Arrows indicate the recombinant proteins, whereas most of minor bands in the rLTBP-4S and rLTBP-4S + eLAP lane are considered to be degradation products of the recombinant protein, as indicated by the Western blot with anti–LTBP-4 antibody (*C*). (*C*–*E*) Antibodies for LTBP-4, elastin and fibulin-5 did not cross-react; 100 ng of each recombinant proteins were subjected to SDS/PAGE under nonreduced conditions, followed by Western blotting with anti–LTBP-4 polyclonal (1:1,000), anti-elastin monoclonal (1:500), and anti–fibulin-5 monoclonal (1:1,000) antibodies.

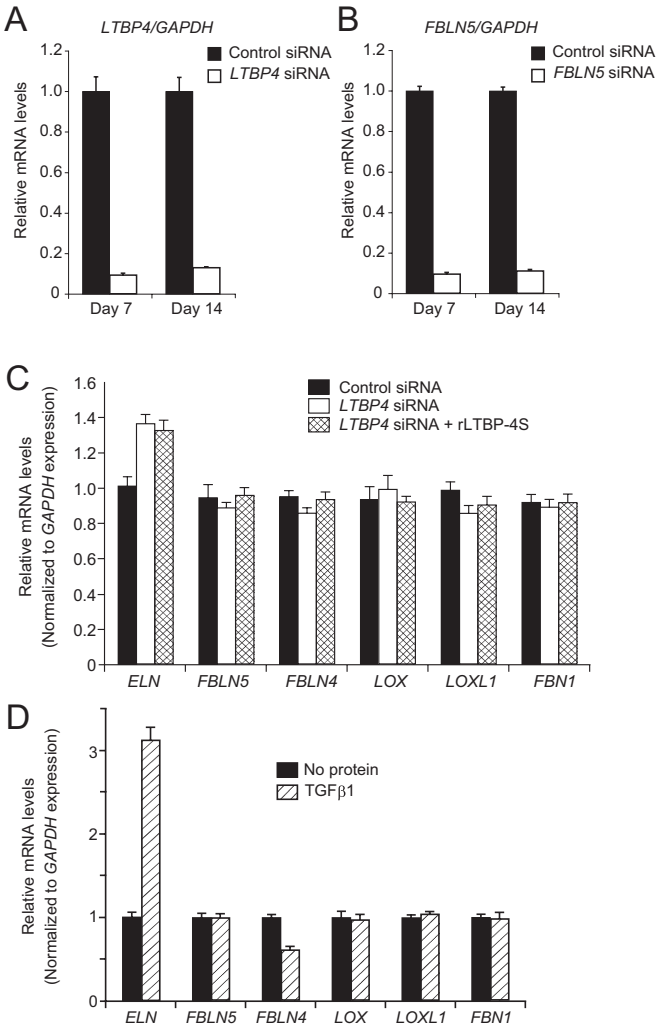

Fig. S5. qPCR analysis of gene knockdown in HDFs. (*A* and *B*) Total RNA from siRNA-transfected HDFs was extracted 7 and 14 d after transfection. cDNA was synthesized and was subjected to real-time PCR to measure mRNA levels of *LTBP4*, *FBLN5*, and *GAPDH*. The mRNA levels relative to untransfected HDFs are shown. Data are presented as means ±SE of four independent experiments. Both *LTBP4* siRNA (*A*) and *FBLN5* siRNA (*B*) effectively suppressed the expression of target genes. Ctrl, control. (*C*) qPCR analysis of elastic fiber–related genes in HDFs transfected with control siRNA or *LTBP4* siRNA, cultured with or without rLTBP-4S (30 nM). Total RNA from siRNA-transfected HDFs was extracted 4 d after transfection. cDNA was synthesized and was subjected to real-time PCR to measure mRNA levels of *ELN*, *FBLN4*, *FBLN5*, *LOX*, *LOXL1*, *FBN1*, and *GAPDH*. The mRNA levels relative to nontransfected HDFs are shown. Data are presented as means ±SE of four independent experiments, each of which were performed in duplicate. (*D*) qPCR analysis of elastic fiber–related genes in HDFs cultured with or without TGF-β1 (1 nM). HDFs were cultured without serum for 48 h and then were added with TGF-β1. Total RNA of HDFs was extracted 12 h after addition of TGF-β1. cDNA was synthesized and was subjected to real-time PCR to measure mRNA levels of *ELN*, *FBLN4*, *FBLN5*, *LOX*, *LOXL1*, *FBN1*, and *GAPDH*. The mRNA levels of HDFs without TGF-β1 were set at 1. Data are presented as means ±SE of three independent experiments.

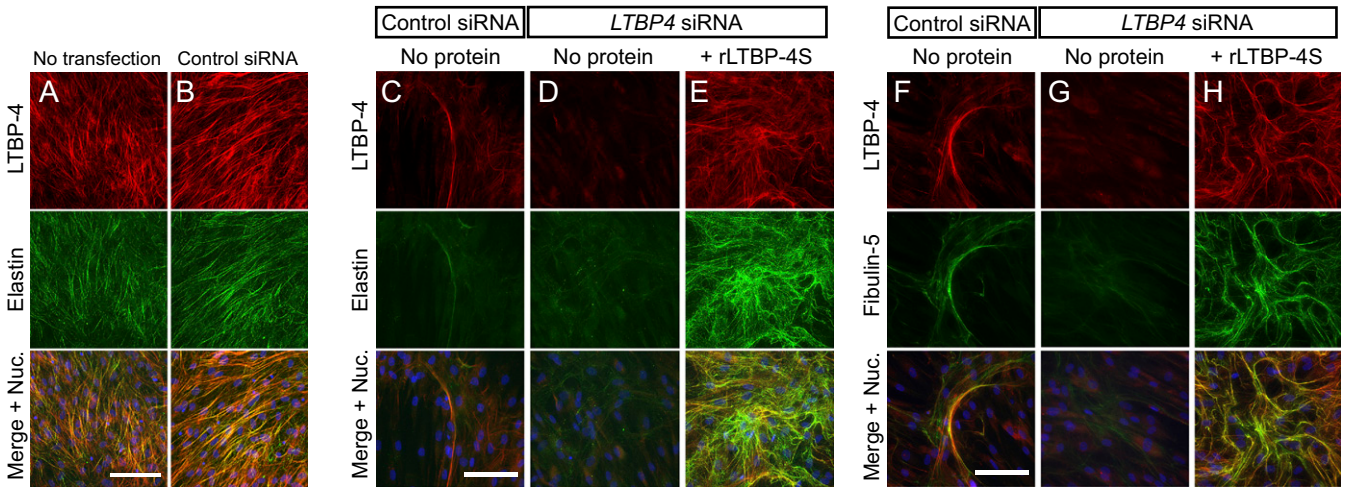

Fig. S6. Elastogenesis in cultures of neonatal and adult HDFs. (*A* and *B*) Control siRNA transfection does not affect elastic fiber assembly. HDFs were transfected with or without control siRNA and cultured in 10% serum-containing media. The cells were fixed 13 d after transfection and stained with anti–LTBP-4 and anti-elastin antibodies. (*C–H*) Normal adult HDFs (106–05 a) were transfected with control siRNA or *LTBP4* siRNA and cultured in 10% serum-containing media supplemented with or without rLTBP-4S (15 nM). The cells were fixed 13 d after transfection and stained with anti–LTBP-4 polyclonal, anti-elastin monoclonal, and anti–fibulin-5 monoclonal antibodies. *Bottom* images were produced by superimposition of the *Top* and *Middle* images, together with Hoechst 33258 nuclear staining. (Scale bar, 100 μm.)

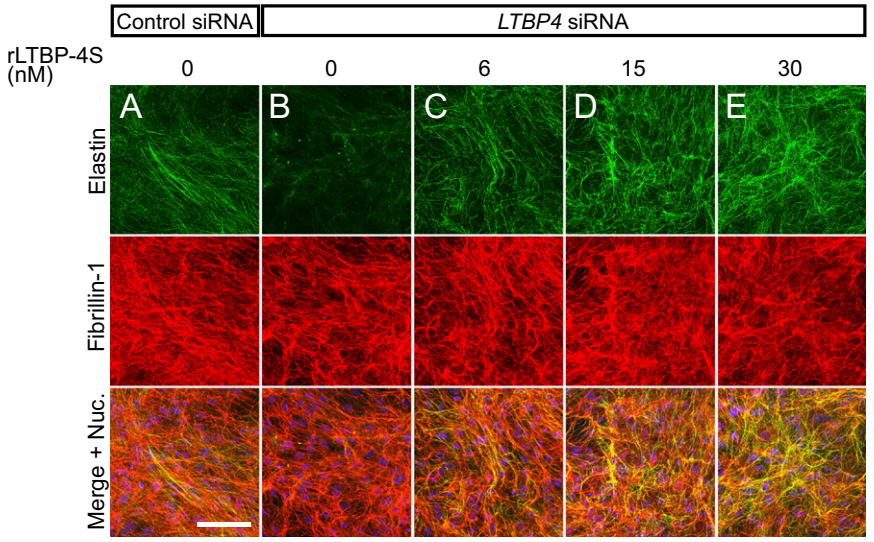

Fig. S7. *LTBP4* knockdown reduced elastin deposition and rLTBP-4S addition augmented elastin deposition, without changing the configuration of fibrillin microfibrils. HDFs were transfected with control siRNA (*A*) or *LTBP4* siRNA (*B–E*) and cultured in 10% serum containing media supplemented with rLTBP-4S as indicated. The cells were fixed 13 d after transfection and stained with anti-elastin and anti–fibrillin-1 antibodies as indicated. *Bottom* images were produced by superimposition of the *Top* and *Middle* images, together with Hoechst 33258 nuclear staining. (Scale bar, 100 μm.)

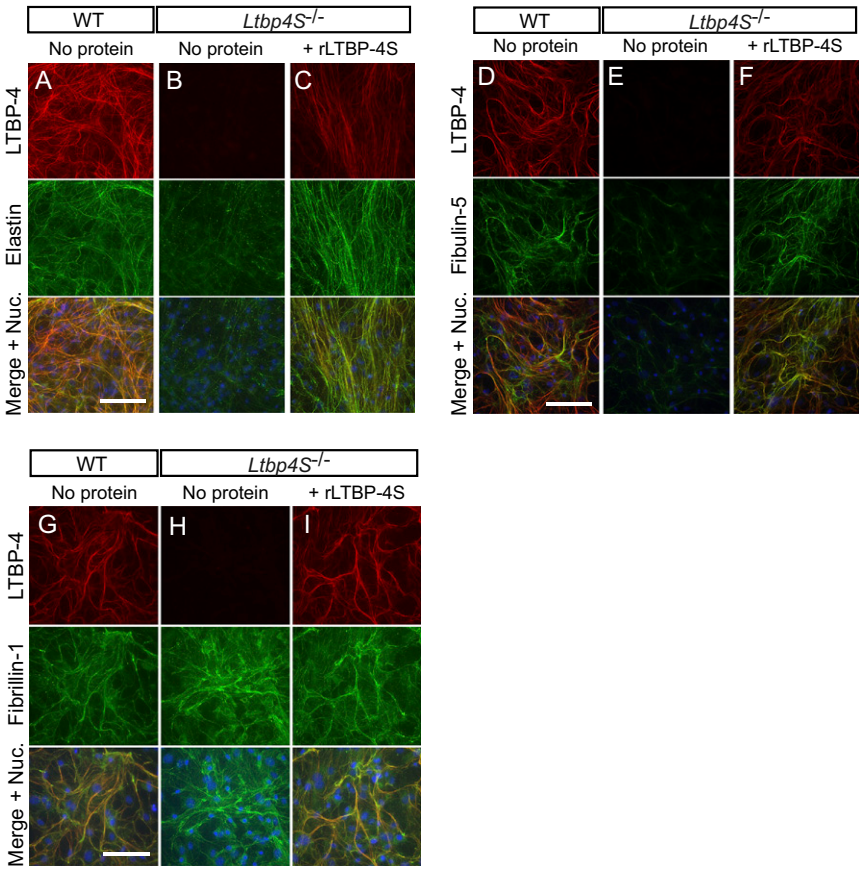

Fig. S8. Elastogenesis in culture of MEFs. (*A–F*) WT MEFs developed an elastic fiber meshwork, whereas *Ltbp4S*$^{-/-}$ MEFs did not. MEFs were cultured in 10% serum containing media on glass coverslips coated with collagen (Cellmatrix; Nitta Gelatin), with or without rLTBP-4S protein (15 nM). The cells were fixed 13 d after plating and stained with goat anti-mouse LTBP-4 polyclonal (R&D Systems), anti-mouse elastin polyclonal (PR385; EPC), and anti–fibulin-5 monoclonal antibodies. (*G–I*) LTBP-4 colocalizes with fibrillin-1. MEFs from WT and *Ltbp4S*$^{-/-}$ embryos were cultured with or without rLTBP-4S protein (15 nM) and were fixed 7 d after plating, followed by immunostaining with rabbit anti–fibrillin-1 polyclonal and goat anti-mouse LTBP-4 polyclonal antibodies. *Bottom* images were produced by superimposition of the *Top* and *Middle* images, together with Hoechst 33258 nuclear staining. (Scale bar, 100 μm.)

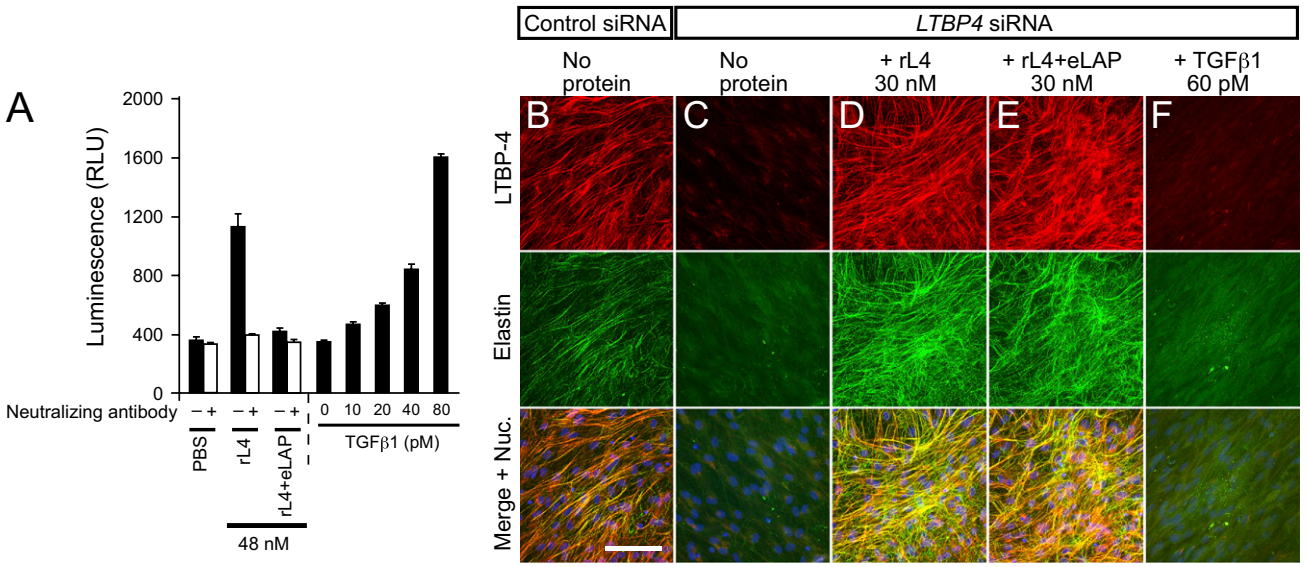

Fig. S9. Elastogenic property of LTBP-4 is not dependent on bound TGF-β. (*A*) TGF-β activity of rLTBP4-S (rL4) was measured using a cell-based TGF-β bioassay system. rL4 was purified from the medium of 293T cells expressing N-terminal 6×histidine (His)-tagged LTBP-4S. rL4 + eLAP was purified from the medium of 293T cells overexpressing His-tagged LTBP-4S and untagged LAP without TGF-β sequence. The fresh media containing each recombinant protein were heat-treated to release active TGF-β and subjected to TGF-β activity assay with or without anti–TGF-β neutralizing antibody (means ±SD, $n = 3$). (*B–F*) HDFs were transfected with control siRNA or *LTBP4* siRNA and cultured for 13 d in 10% serum containing media with or without recombinant protein as indicated. Cells were immunostained with anti–LTBP-4 or anti-elastin antibodies. *Bottom* images were produced by superimposition of the *Top* and *Middle* images, together with Hoechst 33258 nuclear staining. (Scale bar, 100 μm.)

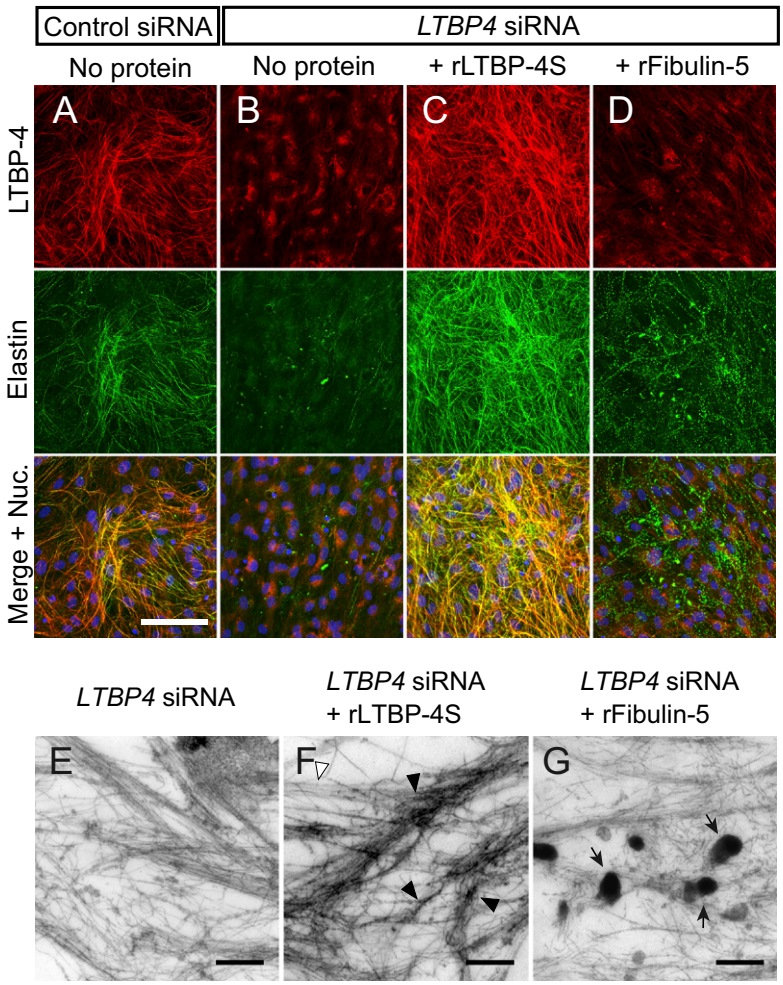

Fig. S10. Aggregated elastin deposition in a punctate pattern in the absence of LTBP-4. HDFs were transfected with control siRNA or *LTBP4* siRNA and cultured for 13 d in 10% serum containing media with or without recombinant proteins as indicated (rLTBP-4S 30 nM or rFibulin-5 60 nM), followed by immunostaining with anti–LTBP-4 and anti-elastin antibodies (*A–D*) or by transmission electron micrography (*E–G*). (*A–D*) *Bottom* images were produced by superimposition of the *Top* and *Middle* images, together with Hoechst 33258 nuclear staining. Addition of rFibulin-5 to *LTBP4* KD cell culture caused increased elastin deposition, but only in a punctate pattern. (Scale bar, 100 μm.) (*E–G*) Elastin was stained by tannic acid. In rLTBP-4S-supplemented cell culture, both elastin-negative microfibrils (open arrowhead) and microfibrils with fine elastin particles (closed arrowheads) were observed. In rFibulin-5-supplemented culture, large granules of aggregated elastin (arrows) were observed. (Scale bars, 500 nm.)