Fibulin-2 and Fibulin-5 Cooperatively Function to Form the Internal Elastic Lamina and Protect From Vascular Injury

Shelby L. Chapman, F.-X. Sicot, Elaine C. Davis, Jianbin Huang, Takako Sasaki, Mon-Li Chu, Hiromi Yanagisawa

Objective—Recent findings on the role of fibulin-5 (Fbln5) have provided substantial progress in understanding the molecular mechanism of elastic fiber assembly in vitro. However, little is known about differential roles of fibulins in the elastogenesis of blood vessels. Here, we generated double knockout mice for Fbln5 and Fbln2 (termed DKO) and examined the role of fibulins-2 and -5 in development and injury response of the blood vessel wall.

Methods and Results—Fibulin-2 is distinctly located in the subendothelial matrix, whereas fibulin-5 is observed throughout the vessel wall. All of the elastic laminae, including the internal elastic lamina (IEL), were severely disorganized in DKO mice, which was not observed in single knockout mice for Fbln2 or Fbln5. Furthermore, DKO vessels displayed upregulation of vascular adhesion molecules, tissue factor expression, and thrombus formation with marked dilation and thinning of the vessel wall after carotid artery ligation-injury.

Conclusions—Fibulin-2 and fibulin-5 cooperatively function to form the IEL during postnatal development by directing the assembly of elastic fibers, and are responsible for maintenance of the adult vessel wall after injury. The DKO mouse will serve as a unique animal model to test the effect of vessel integrity during various pathological insults. (Arterioscler Thromb Vasc Biol. 2009;29:00-00.)

Key Words: internal elastic lamina • vascular remodeling • development • injury

The internal elastic lamina (IEL) is located beneath the endothelium of blood vessels and forms the innermost elastic lamina. The IEL provides elasticity and recoil to the vessel wall, as well as functions as a physical barrier against chemical/mechanical stresses, preventing direct contact of plasma components to smooth muscle cells (SMCs). Several pathological processes have been shown to bring about disruption of the IEL. For example, increased mechanical forces and shear stress associated with angioplasty, enzymatic activation of matrix degrading enzymes in atherosclerosis, and abdominal aneurysms are all associated with disruption of the IEL and progression of vascular disease.1–3 Molecular mechanisms of elastic fiber assembly have begun to be explored by the identification of elastic fiber-associated proteins and their biochemical interactions with elastin or the microfibrillar scaffold.4 Members of the fibulin family of extracellular matrix (ECM) proteins, particularly fibulin-4 and fibulin-5, play essential roles in elastic fiber development.5–7 We demonstrated that fibulin-5 preferentially binds the monomeric form of elastin, but not polymerized elastin.8 Others have shown that fibulin-5 accelerates the self-aggregation process of elastin, called coacervation,9 and fibulin-5 limits maturation of the coacervated elastin.10 Among 5 of the known fibulins tested, fibulin-2 and fibulin-5 exhibit the highest binding affinity to elastin.11 Biochemical interaction assays showed that fibulin-2 also binds numerous basement membrane (BM) proteins including nidogen, laminin, and fibronectin.12 Recently, we have generated knockout mice for the fibulin-2 gene (Fbln2−/−) and unexpectedly found that Fbln2−/− mice do not display significant alterations in elastic fibers in vivo, despite strong tropoelastin binding in vitro.13 In addition, Fbln2−/− embryonic fibroblasts retained the ability to assemble normal fibers of elastin, fibrillin-1, and fibronectin in vitro. Thus, precise roles of fibulin proteins during the assembly process and how they coordinate elastogenesis in different anatomic locations in vivo remain unknown.

In this study, we analyzed the interaction between fibulin-2 and fibulin-5 in vitro and tested their roles in vascular elastogenesis by generating double knockout mice (DKO) for Fbln2 and the fibulin-5 gene (Fbln5). Finally, we examined the effect of disrupted elastic laminae on vessel remodeling using a carotid artery ligation model in DKO mice.

Methods

Mice

Detailed information on Fbln5−/− and Fbln2−/−13 are provided in the supplemental materials (available online at http://atvb.ahajournals.org).
Histology, Immunostaining, and Western Blot Analysis
Detailed information is provided in the supplemental materials.

Electron Microscopy
Aortae were harvested after cardiac perfusion with 3% glutaraldehyde in 0.1 mol/L sodium cacodylate (pH 7.4) and prepared for electron microscopic analysis as described in Supplemental material.

Binding Assays
Recombinant mouse fibulin-2 and fibulin-5 were produced as previously described. Recombinant human tropoelastin was kindly provided by Dr Joel Rosenbloom (University of Pennsylvania, Philadelphia). Detailed methods of solid phase binding assays and surface plasmon resonance assays are provided in the supplemental materials.

Carotid Artery Ligation
Adult male mice between 3 to 8 months of age were used in the study. Ligation of the left carotid artery was performed as previously described. Detailed methods are provided in the supplemental materials.

Morphometric Analysis
Histological sections at level 400 (1.4 mm from the ligature) were digitally captured using Leica DM2000 microscope for comparison between different genotypes. Morphometric analysis was performed with Scion NIH IMAGE Software (National Institutes of Health, Frederick, Md).

Quantitative RT-PCR Analysis
Five to 6 carotid arteries, unligated or ligated, were pooled, and RNA was prepared as described in the supplemental materials.

Statistical Analysis
Data were analyzed using 1-way ANOVA with Bonferroni post hoc tests, t test, or χ2 analyses and a probability value less than 0.05 (P<0.05) was considered statistically significant. Bars indicate the means±SEM unless noted otherwise.

Results
Luminal Surface of IEL Is Maintained in the Adult Fbln5−/− Aorta
Fbln5−/− mice have been established as a mouse model of systemic elastinopathy, involving the vascular system as a major target organ. In adults, Fbln5−/− aortae are elongated and tortuous because of the lack of continuous elastic fibers. However, the defect is not homogeneous throughout the thickness of vessel wall. Disruption of elastic fibers becomes progressively worse toward the adventitia and the IEL is relatively well formed. To evaluate the role of fibulin-5 in the development of the IEL, we used electron microscopy (EM) to examine aortae from postnatal day (P) 1 and P120 wild-type and Fbln5−/− mice. In wild-type aortae, elastic fibers at P1 were not yet continuous, indicating that elastic laminae were being organized during the neonatal period in the wild-type aorta (supplemental Figure IA). Consistent with our previous observations, there was a marked delay in the formation of all elastic laminae in Fbhn5−/− mice (supplemental Figure IB). By P120, however, the surface of the IEL subjacent to the endothelial cells (ECs) was relatively well developed in Fbhn5−/− mice, similar to that of wild-type aortae (compare asterisk in supplemental Figure IC and ID). In contrast, the surface of the IEL adjacent to the first SMC layer and the elastic laminae in the rest of the media remained disrupted, with multiple aggregates of elastin being observed (arrows in supplemental Figure ID). These findings led us to hypothesize that another molecule with a similar biological activity may have compensated for the absence of fibulin-5 in the formation of the IEL.

Binding Profiles Between Fibulin-2, Fibulin-5, and Tropoelastin
Although fibulin-2 interacts with tropoelastin with high affinity in vitro, a recent knockout study has shown that fibulin-2 is dispensable for elastic fiber development in vivo. We speculated that the loss of fibulin-2 was compensated by fibulin-5, and therefore, that fibulin-2 may be able to compensate for the loss of fibulin-5 in IEL formation. We first tested whether fibulin-2 and fibulin-5 physically interact because both fibrinins form homodimers in vitro. Solid phase binding assays indicated that both fibulin-2 and fibulin-5 strongly interact with immobilized tropoelastin (Figure 1A and 1B). Although soluble fibulin-2 showed weak binding to immobilized fibulin-5 (Figure 1A), almost no binding was detected between fibulin-2 and fibulin-5 using the reverse experiment (Figure 1B). This was confirmed by surface plasmon resonance assays where the calculated dissociation constant (Kd) between fibulin-2 and fibulin-5 was attributable to the higher dissociation rate constant of the fibulin-2 and tropoelastin interaction. These data from the 2 different binding assays indicate that fibulin-2 and fibulin-5 are unlikely to interact in vitro.

Generation of DKO Mice
Because the IEL continues to develop after birth, we speculated that fibulin-2 might compensate for the absence of fibulin-5 in establishing the IEL in Fbhn5−/− mice. We first examined whether there was a compensatory upregulation of fibulin-2 in the Fbhn5−/− aorta by Western blot analysis (Figure 2A). The bands corresponding to fibulin-2 (arrowhead and asterisk), which were absent in Fbhn2−/− (also see supplemental Figure I for deletion of Fbhn2), were modestly upregulated in Fbhn5−/−/.
Aorta compared to wild-type aortae (Figure 2Aa). Interestingly, fibulin-5 was significantly upregulated in Fbln2−/− aortae compared to wild-type (Figure 2Ab and 2Ac). To further test the hypothesis that fibulin-2 compensates for fibulin-5 in the formation of IEL, we generated double knockout mice for Fbln2 and Fbln5.

In agreement with recently reported findings, fibulin-2 and fibulin-5 were absent in DKO mice. Their surface was similar to that observed in single knockout animals (Figure 2Bd and 2Bh). Taken together, these data indicate that protein localization of fibulin-2 and fibulin-5 are independent of each other in vivo.

To confirm the absence of fibulin-2 and fibulin-5 in the DKO aorta, immunohistochemistry was performed using antifibulin-2 and antifibulin-5 antibodies (Figure 2B). Consistent with previous data, fibulin-2 localized most strongly to the IEL and fibulin-5 was observed throughout the aortic wall of adult wild-type mice with intense staining (Figure 2Ba and 2Be). Localization of fibulin-2 and fibulin-5 was not altered in single Fbln5−/− and Fbln2−/− mice, respectively (Figure 2Bc and 2Bf), and both proteins were absent from DKO (Figure 2Bd and 2Bh). Taken together, these data indicate that protein localization of fibulin-2 and fibulin-5 are independent of each other in vivo.

The IEL Is Markedly Disrupted in DKO Aorta

We next examined the sublumenal region of the aorta at the ultrastructural level. In the wild-type aorta, a solid IEL was formed under the EC layer and a small extracellular region was seen between IEL and ECs (Figure 3A). The basement membrane of the ECs was tight to the endothelium and situated close to the underlying IEL. The Fbln2−/− IEL was indistinguishable from wild-type (Figure 3B). In the Fbln5−/− aorta, the surface of the IEL adjacent to the ECs was solid, however, small disruptions of the IEL were observed on the SMC side of the IEL (arrowheads in Figure 3C). In contrast to wild-type aortae, the BM in Fbln5−/− animals was separated from the endothelium and the subendothelial region was wider (arrows in Figure 3C). In DKO aortae, we observed 2 remarkable abnormalities. First, the IEL was markedly disrupted with core of the elastic lamina being severely thinner than that from either the wild-type or single knockout animals (asterisk in Figure 3D), suggesting that fibulins-2 and -5 aid in the assembly of IEL. Second, the subendothelial region was increased significantly and the BM was clearly visible and separated from the endothelium and underlying IEL (arrows in Figure 3D). Two possibilities can be
suggested from this observation: (1) changes in the subendothelial ECM occur after the formation of the IEL and BM which influence the eventual organization of these structures and the association of the EC with the underlying matrix, or (2) lack of fibulins-2 and -5 in subendothelial matrix affects the stabilization of EC-ECM interactions, because fibulin-2 binds numerous BM proteins and mouse fibulins-2 and -5 each contain a RGD motif that mediates RGD-dependent integrin binding.

**Altered Expression of BM Proteins in DKO Aorta**

To determine whether mislocalization or altered expression of BM proteins is involved in alteration of subendothelial ultrastructure, we examined BM proteins in DKO mutants. Using immunofluorescence staining, the EC layer was visualized with CD31 (Figure 4A and 4B). Laminin γ1 staining was observed uniformly throughout the vessel wall in the wild-type aorta (Figure 4C). In contrast, the staining was increased at the luminal surface of the aorta in the DKO mouse (Figure 4D). Collagen IV staining was slightly increased in the DKO aorta at the luminal surface when compared to the wild-type aorta (Figure 4E and 4F). Next, we examined whether the structural changes of IEL led to activation of ECs by staining for the adhesion molecules, ICAM-1 and VCAM-1. These molecules are known to be upregulated in atherosclerosis and other pathological insults. As shown in Figure 5G and 5H, ICAM-1 staining was more intense in DKO compared to wild-type vessels, suggesting that DKO ECs were affected by the disrupted contact with the BM. VCAM-1 staining was unchanged in DKO aorta (data not shown). We then asked whether the altered composition of ECM proteins affected differentiation of vascular SMCs. Staining for SM myosin heavy chain and α-SM actin, a marker of late and early differentiated SMCs, respectively, was indistinguishable between wild-type and DKO aorta, although the alignment of SMCs was disrupted and the number of lamellar units was increased in DKO aorta (supplemental Figure III).

**DKO Vessels Display Abnormal Vascular Remodeling After Carotid Artery Ligation-Induced Injury**

Finally, we determined whether a compromised IEL in addition to disrupted medial elastic laminae would further affect the response to vascular injury or vessel remodeling in vivo by using a carotid artery ligation-induced injury model. The left carotid artery was ligated proximal to the bifurcation and maintained for 28 days, a time-point when neointima formation has become most prominent. Serial transverse sections were analyzed from 1.0 mm proximal to the ligature (designated as level 0) to 1.9 mm (level 900), and morphometric analysis was performed at level 400. Elastin staining of unmanipulated vessels from all genotypes did not reveal any differences in a vessel diameter (supplemental Figure IV). On injury, wild-type and Fbn2−/− arteries showed little neointima and the elastic laminae exhibited a typical undulating structure (Figure 5Aa, 5Ab, 5Ba, and 5Bb). Consistent with previous observations, Fbn5−/− vessels developed a severe neointima (Figure 5Ac). In contrast, despite a modest neointima being formed, DKO vessels developed a severe, organized thrombus that occupied an abnormally enlarged lumen (Figure 5Ad). Elastic laminae in both Fbn5−/− and DKO vessels were distended as indicated by the absence of undulations (Figure 5Bc and 5Bd). Whereas both wild-type and Fbn2−/− vessels underwent constrictive (negative) remodeling after 28 days (Figure 5C, WT and Fbn2−/−), Fbn5−/− vessels showed minimal negative remodeling (Figure 5C, Fbn5−/−) and the IEL perimeter was only marginally decreased. Remarkably, the DKO vessels showed an enlarged lumen (outward remodeling), greatly exceeding the original perimeter of unmanipulated vessels (Figure 5C, DKO). Comparisons of medial wall thickness among genotypes revealed that the media was extremely thin in vessels from DKO mice after injury (Figure 5D). The remodeling of postinjured carotid arteries assessed after perfusion fixation showed similar results to those obtained with immersion fixation only (supplemental Figure V). These data indicate that DKO vessels were unable to undergo injury-induced vascular remodeling.

When the intima/media ratio was compared, DKO vessels showed less neointima but a marked increase in thrombus formation compared with wild-type vessels (Figure 5F, marked in red). Seven of 8 vessels from DKO animals developed thrombus that occupied more than 50% of the lumen, whereas only 1 of 6 vessels from Fbn5−/− mice developed mixed a lesion consisting of thrombus and neointima (P=0.026, χ², supplemental Figure VIA and VI3B). One DKO vessel developed severe neointima as Fbn5−/− vessels, but the vessel diameter was even more increased in the DKO vessel compared to Fbn5−/− vessels (supplemental Figure VIC and VD).

It has been shown that positive remodeling is associated with structural changes of the media and adventitia, including medial and adventitial breakdown, together with plaque components in a rabbit vascular atherothrombosis model. Therefore, we evaluated the changes in the adventitia in all genotypes. Whereas no difference was detected in adventitia thickness among unmanipulated vessels (data not shown), adventitia area was significantly increased in injured DKO vessels. The ratio between adventitia to total vessel area, however, was unchanged in DKO.
Figure 5. Vascular remodeling after carotid artery ligation–induced injury. A and B, H&E and Harts’ staining of transverse sections of injured vessels at level 400. Bars in A and B indicate 100 μm and 80 μm, respectively. Arrow in Bd indicates disruption of the IEL in the DKO. C, D, E, and F, Morphometric analysis of carotid arteries from unmanipulated, injured, and contralateral vessels at level 400. In F, red indicates formation of a thrombus that occupied more than 50% of lumen. In A through F, analysis was done 28 days after the injury. G, Early changes of endothelial cells and SMCs after the ligation-induced injury at indicated time point. Arrows in Gi indicate PECAM-1 expression in endothelial cells and thrombus in DKO vessels. Bars indicate 50 μm.
In contrast, the ratio was significantly increased in the injured \textit{Fbln5}\textsuperscript{−/−} vessels (Figure 5E).

To gain insight into the pathological changes that lead to thrombus formation in \textit{DKO} vessels, we harvested wild-type and \textit{DKO} vessels at 2 days and 7 days after ligation and examined the expression of vascular adhesion molecules by immunostaining (Figure 5G). PECAM-1 was downregulated in both wild-type and \textit{DKO} vessels 2 days after injury compared to contralateral vessels (Figure 5Ga-5Gd). On day 7, PECAM-1 expression was regained in ECs of wild-type injured vessels but to a lesser extent in \textit{DKO} vessels. PECAM-1 was also detected in the forming thrombus in \textit{DKO} vessels (Figure 5Gf, arrows). ICAM-1 was not detected in wild-type injured or contralateral vessels (supplemental Figure VIIa, VIIc, VIIe, and VIIg), however expression was observed in ECs of injured \textit{DKO} vessels and contralateral vessels at 2 days (supplemental Figure VIIb, VIId, VIIf, and VIIh). VCAM-1 was upregulated in the \textit{DKO} vessels at 2 days after injury and the expression was much stronger and extended to the medial layers at 7 days (Figure 5Gj and 5Gn) compared to wild-type injured vessels (Figure 5Gi and 5Gm).

We finally asked whether the \textit{DKO} injured vessels affected the expression of tissue factor (TF), which is a key molecule involved in extrinsic coagulation pathway and shown to mediate arterial injury-induced thrombosis.\textsuperscript{21} Quantitative RT-PCR analysis of uninjured carotid arteries from \textit{DKO} mice showed significantly lower expression of \textit{TF} transcripts compared to wild-type arteries, whereas von Willebrand factor (vWF), a key molecule in the intrinsic coagulation pathway, showed similar expression (supplemental Figure VIII). Interestingly, however, carotid arteries harvested at 2 days postinjury from \textit{DKO} mice showed upregulation of \textit{TF} compared to the wild-type injured arteries. Transcripts for vWF were comparable between wild-type and \textit{DKO} vessels after injury. Taken together, these data indicate that IEL disruption has a profound effect on the activation of vascular cells after arterial injury, leading to a permissive environment for thrombus formation.

Discussion
Elastic fibers are formed by the assembly of tropoelastin monomers onto a microfibrillar scaffold and subsequently crosslinked to form an insoluble elastin polymer.\textsuperscript{22,23} Whereas \textit{Fbln5} expression is detected throughout the vessel wall during embryogenesis,\textsuperscript{24} \textit{Fbln2} is expressed only in the SMC layers in midgestation. No expression for \textit{Fbln2} is detectable in ECs until approximately E18. However, fibulin-2 becomes prominently localized to the BM region of ECs in the early postnatal period, which coincides with a period of active elastic fiber assembly.\textsuperscript{16}

In adult \textit{Fbln5}\textsuperscript{−/−} mice, a comparable amount of assembly was seen on the luminal side of the IEL compared to age-match wild-type mice. However, the surface of the IEL adjacent to SMCs and the medial and external elastic lamina (EEL) never assembled properly. This suggests that the mechanism of IEL formation is distinct from other elastic laminae, and this mechanism is maintained even in the absence of fibulin-5. A dramatic disruption of the IEL in the \textit{DKO} aorta clearly demonstrates that fibulin-2 and fibulin-5 cooperatively function to form IEL during development. Although we have not examined whether fibulin-2 regulates coacervation or maturation of tropoelastin, it is likely that fibulin-2 has a similar molecular function as fibulin-5 and that fibulin-2 can compensate for fibulin-5 and facilitate assembly of the IEL when fibulin-5 is absent. Taken together, it implies that a tissue-specific elastogenesis mechanism involving different members of fibrulins may exist in vivo.

It is interesting to note that \textit{DKO} vessels do not develop spontaneous aneurysms despite a severe developmental defect of the IEL and medial elastic laminae. We observed upregulation of major BM proteins, including laminin and collagen IV in \textit{DKO} vessels. Because laminin is shown to attenuate EC response to shear stress, such as nuclear translocation of NF-κB and activation of C-Jun NH₂-terminal kinase (JNK),\textsuperscript{25,26} the changes in subendothelial matrix composition in \textit{DKO} vessels may influence EC stability in noninjured conditions. After injury, however, \textit{DKO} vessels develop severe thrombus with thinning of the medial wall and a marked enlargement of the vessel diameter. Unlike a wire withdrawal injury model, ligation-induced injury causes stasis of blood flow without directly damaging the ECs. However, stasis and hypoxia in ligated vessels can induce fibrin deposition onto ECs through accumulation of inflammatory cells and activated platelets.\textsuperscript{27} We observed upregulation of VCAM-1 and ICAM-1 from 2 days after the ligation in \textit{DKO} vessels, suggesting that attachment of ECs onto an intact IEL provides protection and stabilization of ECs during vascular injury. In addition, an increase in the \textit{TF} transcripts in \textit{DKO} arteries on injury is compatible with the thrombotic phenotype in \textit{DKO} mice. Because SMC-derived TF has recently been shown to be critical for thrombus formation after arterial injury, a compromised IEL may further facilitate production and interaction of TF with plasma components and lead to the activation of the coagulation cascade.

Morphological changes of the IEL in \textit{DKO} vessels are much more severe than those in \textit{Fbln5}\textsuperscript{−/−} vessels. A previous report suggests that damage involving the EEL can be a more potent stimulus for neointima formation than a lesion only involving the IEL.\textsuperscript{1} MMP upregulation was shown to correlate with the extent of loss of elastic content and architecture in Marfan mouse models.\textsuperscript{28} Our present study highlights that the IEL also plays a role in determining vessel integrity during injury by providing structural stability to the vessel wall.

In injured \textit{DKO} vessels, the total adventitial area was significantly increased compared with wild-type or \textit{Fbln2}\textsuperscript{−/−} vessels, however \textit{DKO} vessels failed to maintain a normal diameter. It has been reported that in the angiotensin II-induced ApoE\textsuperscript{−/−} aorta, the intact vessel is surrounded by remodeled adventitia, whereas a break in medial layers is accompanied by a thin adventitial layer, suggesting a protective role of adventitia in vessel remodeling.\textsuperscript{29} In the current study, the ratio between adventitia and total vessel area was significantly increased in \textit{Fbln5}\textsuperscript{−/−} vessels compared to \textit{DKO} vessels, and \textit{Fbln5}\textsuperscript{−/−} vessels showed less abnormal remodeling compared to \textit{DKO} vessels. Thus, adequate adventitial thickening in the presence of an intact IEL may be critical for maintaining vessel remodeling during injury.

We have previously proposed 2 mutually compatible mechanisms for accelerated neointima formation in \textit{Fbln5}\textsuperscript{−/−} vessels.
after injury. One is attributable to a developmental defect of the elastic laminae and the inability to correctly assemble elastic fibers within the neointima, and the other is attributable to the lack of an inhibitory effect of fibulin-5 on proliferation and migration of SMCs. In vitro data by others also suggests that fibulin-5 inhibits SMC recruitment and EC proliferation. Fibulin-2, on the other hand, was proposed to increase SMC migration of SMCs. In vitro data by others also suggests that lack of an inhibitory effect of fibulin-5 on proliferation and fibers within the neointima, and the other is attributable to the elastic laminae and the inability to correctly assemble elastic.

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Disclosures
None.

References
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Supplemental Material

Fibulin-2 and fibulin-5 cooperatively function to form the internal elastic lamina and protect from vascular injury

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Detailed Methods

Mice. Fbln5−/− mice were maintained on a C57BL6 x 129/SvEv hybrid background and kept on a 12 h/12 h light/dark cycle under specific pathogen free condition ¹. Fbln2−/− mice were maintained on a 129 background prior to intercross with Fbln5+/− mice ². Wild-type or heterozygous littermates were used as control. All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committees.

Histology. H&E staining was used for routine histological observation, and modified Hart's staining for visualization of elastic fibers. Immunostaining was performed using at least three animals per genotype. Detailed methods are provided in Supplemental material.

Western blot analysis. Aortae were harvested from 3-month old mice, minced and heated in 2 x SDS-PAGE sample loading buffer for 5 min at 95°C. Five to 10 µl of each sample was subject to Western blot analysis using anti-fibulin-2 (1:2000), anti-fibulin-5 (1:200), or anti-α-tubulin (1:5000, Sigma-Aldrich, Inc.).
Electron microscopy. Aortae harvested following cardiac perfusion were dissected into small segments and left in fixative (3% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4)) overnight at 4°C. After extensive washing in 0.1 M sodium cacodylate buffer, the tissues were sequentially treated with osmium tetroxide, tannic acid and uranyl acetate, then dehydrated and embedded in Epon as previously described. Thin sections (60 nm) were counterstained with methanolic uranyl acetate and lead citrate and viewed using a Tecnai 12 transmission electron microscope at 120 kV.

Immunostaining. Cryosections were fixed in 4% paraformaldehyde/PBS for 20 min at room temperature (RT), then blocked in 1% BSA/2% normal goat serum (NGS) for 20 min at RT. Primary antibodies were diluted in 1% BSA/2% NGS and incubated on sections overnight at 4°C. After washing 5 x 5 min in PBS, sections were incubated with fluorescein conjugated goat anti-rabbit secondary antibody (1:50, Vector Laboratories) or Alexa fluor 594 conjugated donkey anti-rabbit secondary antibody (1:50, Invitrogen) for 1 hr at RT. After washing with PBS, sections were mounted in Vectashield with DAPI (Vector Laboratories). Sections were viewed using a fluorescence microscope (DMRXE, Leica) or a confocal laser scanning microscope (LSM 510-V3.2, Carl Zeiss MicroImaging, Inc.). Antibodies used in this study were: anti-fibulin-5 (1:500), anti-fibulin-2 (1:200), anti-collagen IV (1:200, Chemicon International), anti-lamininγ1 (1:50), anti-α-SM-actin (1: 500, Sigma-Aldrich, Inc.), anti-SM myosin heavy chain (1:100, Sigma-Aldrich, Inc.), anti-CD31 (1:50, BD Biosciences)

**Binding Assays.** Solid-phase binding assays were performed as previously described with fibulin-2 as the soluble ligand and tropoelastin or fibulin-5 in solid-phase, or with fibulin-5 as soluble ligand and tropoelastin or fibulin-2 in solid-phase. For the solid-phase, recombinant proteins (0.5 µg/well) were coated on the plate. Surface plasmon resonance assays were performed with BIAcore 1000 (BIAcore AB). Fibulin-2, fibulin-5 and tropoelastin were diluted to 200 µg/ml in 0.1M sodium acetate, pH 4.0 and covalently coupled to CM-5 sensor chips. Immobilization levels were adjusted to 1000-3000 resonance units (RU). Binding assays were performed with ligands (5-500 nM) dissolved in TBS containing 0.005% P20 surfactant (BIAcore AB) and 2mM CaCl₂ at 25°C. Kinetic constants were calculated by non-linear fitting of the association and dissociation curves according to a 1:1 model following the manufacturer's instructions.

**Carotid artery ligation.** After the ligation, both right and left carotid arteries were harvested at 48 h (n=4 per group), 7 days (n=4 per group) or 28 days (n=7, 5, 6, and 8 for WT, Fbln2⁻/⁻, Fbln5⁻/⁻ and DKO, respectively) post-injury en bloc without perfusion, followed by immersion fixation in 4% paraformaldehyde in PBS for 16-24 hrs at 4°C. To test the effect of immersion fixation versus perfusion fixation on tissue preservation and structure, cardiac perfusion was performed at 28 days after the ligation (n=4, 5, and 3 for WT, Fbln5⁻/⁻ and DKO, respectively) with PBS followed by 4% paraformaldehyde. No differences were observed thus validating
the use of immersion fixation in this study. The left and right carotid arteries were embedded in paraffin, and positioned to obtain transverse sections, with 1mm below (proximal to) the suture designated as level 0. For unmanipulated vessel measurements, both right and left carotid arteries were harvested without performing injury.

**qPCR analysis.** RNA was isolated using a RNeasy Plus Micro Kit (QIAGEN). One µg of total RNA was subjected to reverse transcription using superscript III reverse transcriptase (Invitrogen). SYBR Green was used for amplicon detection and gene expression was normalized to expression of Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH). PCR reactions were carried out in the CFX96 real-time PCR detection system (Bio-Rad) with one cycle of 3 min at 95°C, then 40 cycles of 10 sec at 95°C and 1 min at 60°C. Primer sequences are provided in Supplemental Table 1.
Reference


Supplemental Figure legends

Figure I. The lumenal side of IEL is preserved in the Fbln5−/− aorta.
Electron micrographs of P1 (A, B) and P120 (C, D) wild-type (WT, A, C) and Fbln5−/− (B, D) aortae. At P1, elastic fibers are being organized in WT aorta (arrows in A). At P120, continuous elastic laminae are observed in the WT aorta (arrows in C). In the Fbln5−/− aorta, elastic fibers remain disrupted in medial elastic laminae (single arrow in D). The lumenal side of IEL adjacent to the endothelial cells (en) (asterisk in D) is complete, whereas the surface of the IEL adjacent to the SMCs is disorganized (double arrows in D). Bars indicate 3 μm. Asterisk in A-C indicates the IEL.

Figure II. Genomic PCR detecting WT (upper gel) and mutant alleles (lower gel) of Fbln2. PC; positive control, NC; negative control.

Figure III. Evaluation of SMCs in DKO aorta. Immunostaining of cryosections prepared from the descending thoracic aorta from WT (A, C) and DKO (B, D) mice at 3-months of age stained SM-MHC (A, B), and α-SM actin (C, D). Bars indicate 50 μm.

Figure IV. Elastin staining of unmanipulated carotid artery harvested from wild-type, Fbln2−/−, Fbln5−/− and DKO mice. Bars indicate 100 μm.
Figure V. Morphometric analysis of post-injured carotid arteries assessed following perfusion fixation (left, + perfusion) or immersion fixation (right, - perfusion). Ligated (inj) and contralateral (ctl) carotid arteries were harvested on 28 days after ligation injury. A. IEL perimeter of the vessels. B. Medial wall thickness. C. Intima to medial vessel area ratio from perfusion-fixed injured vessels is plotted.

Figure VI. Elastin staining of injured vessels showing various pathological changes 28 days after ligation. (A, B) Fbln5−/− and (C, D) DKO vessels. Arrows in A and C indicate thrombus formation. DKO vessels display dilation of the vessel wall. Bars indicate 100 µm.

Figure VII. ICAM-1 staining of injured and contralateral vessels harvested at 48 h and 7 days after the ligation from WT (a, c, e, g) and DKO (b, d, f, h) mice. Note upregulation of ICAM-1 in DKO vessels. Bars indicate 50 µm.

Figure VIII. qPCR analysis of TF and vWF in uninjured or 48h post-ligated carotid arteries from WT and DKO mice. Transcript levels were compared between WT and DKO vessels after normalized to internal control (GAPDH).
Supplemental Table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>TF (Tissue factor)</td>
<td>CATGGAGACGGAGACCAACT</td>
<td>CCATCTTGTCAAACCTGCTGA</td>
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<tr>
<td>Vwf (Von Willebrand Factor)</td>
<td>GCTTGAACTGTGAGACGAGGAGG</td>
<td>TGACCCAGCAGCAGGATGAC</td>
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<tr>
<td>GAPDH</td>
<td>AGTATGACTCCACTCAGGCAA</td>
<td>TCTCGCTCCTGGAAGATGGT</td>
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**Supplemental Table 2.**
Kinetic rate constants and dissociation equilibrium constants of fibulins binding to tropoelastin

<table>
<thead>
<tr>
<th>Immobilized ligand</th>
<th>Soluble ligand</th>
<th>$k_{diss} \times 10^4$ (s$^{-1}$)</th>
<th>$k_{ass} \times 10^{-4}$ (M$^{-1}$s$^{-1}$)</th>
<th>Kd (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tropoelastin</td>
<td>fibulin-2</td>
<td>2.1 ± 0.7</td>
<td>14.9 ± 8.1</td>
<td>0.9 ± 0.2</td>
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<tr>
<td></td>
<td>fibulin-5</td>
<td>35 ± 5</td>
<td>6.2 ± 1.7</td>
<td>59 ± 9</td>
</tr>
</tbody>
</table>

Soluble ligands were used in the concentration range 50 to 1000 nM. Mean values ± S.D. are based on three or four independent determinations.