Abnormal mechanosensing and coflin activation promotes the progression of ascending aortic aneurysms in mice

Yoshito Yamashiro,† Christina L. Papke,† Jungsil Kim,† Lea-Jeanne Ringuette,† Qing-Jun Zhang,‡ Zhi-Ping Liu,‡ Hamid Mirzaei,§ Jessica E. Wagenseil,¶ Elaine C. Davis,¶ Hiromi Yanagisawa1,‡

Smooth muscle cells (SMCs) and the extracellular matrix (ECM) are intimately associated in the aortic wall. Fbln4SMKO mice with an SMC-specific deletion of the Fbln4 gene, which encodes the vascular ECM component fibulin-4, develop ascending aortic aneurysms that have increased abundance of angiotensin-converting enzyme (ACE); inhibiting angiotensin II signaling within the first month of life prevents aneurysm development. We used comparative proteomics analysis of Fbln4SMKO aortas from postnatal day (P) 1 to P30 mice to identify key molecules involved in aneurysm initiation and expansion. At P14, the actin depolymerizing factor coflin was dephosphorylated and thus activated, and at P7, the abundance of slingshot-1 (SSH1) phosphatase, an activator of coflin, was increased, leading to actin cytoskeletal remodeling. Also, by P7, biomechanical changes and underdeveloped elastic lamina–SMC connections were evident, and the abundance of early growth response 1 (Egr1), a mechanosensitive transcription factor that stimulates ACE expression, was increased, which was before the increases in ACE abundance and coflin activation. Postnatal deletion of Fbln4 in SMCs at P7 prevented coflin activation and aneurysm formation, suggesting that these processes required disruption of elastic lamina–SMC connections. Phosphoinositide 3-kinase (PI3K) is involved in the angiotensin II–mediated activation of SSH1, and administration of PI3K inhibitors from P7 to P30 decreased SSH1 abundance and prevented aneurysms. These results suggest that aneurysm formation arises from abnormal mechanosensing of SMCs resulting from the loss of elastic lamina–SMC connections and from increased SSH1 and coflin activity, which may be potential therapeutic targets for treating ascending aortic aneurysms.

INTRODUCTION

Thoracic aortic aneurysms (TAAs) can be either nonsyndromic or syndromic and are associated with a high risk of mortality from dissection and/or rupture. Substantial efforts have gone into identifying the genes and signaling pathways involved in TAAs in humans (1). Genetic analysis of TAA has revealed that mutations in extracellular matrix (ECM) proteins, including fibrillin-1 (2), type III α1-collagen (3), and fibulin-4 (4, 5), lead to the formation of aneurysms, along with various symptoms including skeletal, cutaneous, and connective tissue defects. Furthermore, activation of transforming growth factor β (TGFβ) in the vascular wall, by loss of proper tethering of TGFβ and/or abnormal compensatory increase in TGFβ activity, leads to aortic aneurysms in Marfan syndrome and related disorders (6–8). Whether altered signal transduction is involved in other types of aneurysms characterized by compromised ECM is not completely understood. The ECM also affects the phenotype of smooth muscle cells (SMCs) (9, 10).

Mutations in genes encoding SMC contractile proteins, including SMC-specific myosin heavy chain (Myh11) (11) and α-smooth muscle actin (ACTA2) (12), and type I cyclic guanosine monophosphate (cGMP)–dependent protein kinase (PRKG1) (13) are responsible for nonsyndromic familial TAAs and dissection in humans. A heterozygous mutant allele of MYH11 or ACTA2 might serve as a dominant negative to impair the force-generating machinery of SMCs (11), and a gain-of-function mutation of PRKG1 may reduce SMC contractility (13). Both mutations affect the contraction of SMCs in the ascending aorta, a region where cells are constantly exposed to high outflow pressure.

We have previously shown that SMC-specific Fbln4 knockout mice (Fbln4SMKO) develop ascending aortic aneurysms characterized by disruption of the elastic laminae and increased proliferation of SMCs (9). Proliferation of SMCs precedes aneurysm formation and is associated with increased phosphorylation of extracellular signal–regulated kinase (ERK) and decreased expression of SMC differentiation markers in the aortas of the mutant mice. In addition, abnormal increases in angiotensin-converting enzyme (ACE) abundance and subsequent activation of angiotensin II (AngII) signaling in the aortic wall are responsible for driving the aneurysm phenotype (14). In this model, aneurysms are completely prevented by administration of an ACE inhibitor or AngII type 1 receptor (AT1R) blocker (ARB) within the first month of life. ARB treatment initiated after establishment of the aneurysms does not reverse the aneurysm phenotype, indicating that the signal(s) required for maintenance of aneurysms may be independent of AngII-AT1R. The precise molecular events that occur within this therapeutic time window, however, have not been determined. The aim of the present study, therefore, was to determine the molecular
pathways involved in initiation and expansion of aneurysms and to identify key molecules that potentially connect ECM defects, AngII signaling, and aneurysm formation in Fbln4SMKO mice.

RESULTS

Proteins with differential abundance were identified in Fbln4SMKO aortas during initiation and expansion of aneurysms

To determine the time points involved in the increase in AngII signaling and phenotypic changes in SMCs, we first examined the abundance of ACE and the phosphorylation of ERK in control and Fbln4SMKO aortas from postnatal day (P) 1 to P90. ACE amounts began to increase at P7 in Fbln4SMKO aortas and reached a twofold increase at P30 compared to control (Fig. 1A and fig. S1). Consistent with previous findings, the phosphorylation of ERK was increased at P30. In addition, expression of SMC differentiation marker genes was either maintained [Acta2, which encodes α-smooth muscle actin (SMA)] or increased (Cnn1, which encodes calponin 1; Myh11, which encodes the heavy chain of smooth muscle myosin; SM22, which encodes smooth muscle protein 22-α; Myocd, which encodes myocardin; and Srf, which encodes serum response factor) 2- to 3.5-fold in the Fbln4SMKO aorta at P1, followed by a sharp decrease after P7 (Fig. 1B), suggesting that Fbln4-deficient SMCs rapidly dedifferentiated after birth. On the basis of these data, we concluded that the first 30 days of the postnatal period is a critical time for the initiation and expansion of aneurysms.

Fig. 1. Proteomic analysis of ascending aortas during initiation and expansion of aneurysms. (A) Representative Western blots of ascending aortas from control (CTRL) and Fbln4SMKO (SMKO) mice. The experiment was performed three times with different pools of animals. The numbers of aortas used per genotype in each time point are as follows: P1, n = 5 to 12; P7, n = 5 to 15; P14, n = 5 to 12; P30, n = 3 to 7; and P90, n = 2 to 6. See fig. S1 for quantification. (B) Quantitative polymerase chain reaction (qPCR) analysis of SMC-specific genes from ascending aortas of CTRL (pooled P1, n = 12; P7, n = 9; and P30, n = 12) and Fbln4SMKO (pooled P1, n = 12; P7, n = 18; P14, n = 9; and P30, n = 11) mice performed in technical triplicate. (C) Representative two-dimensional differential gel electrophoresis (2D-DIGE) using entire aortas (for P1, n = 4 per genotype) or ascending aortas (P7, n = 7; P14, n = 5; and P30, n = 3 per genotype). Proteins with increased abundance in the Fbln4SMKO aortas appear in red, proteins with decreased abundance appear in green, and those with similar abundance appear in yellow. Circled spots with numbers indicate more than twofold changes between CTRL and Fbln4SMKO in three independent experiments. (D) Heat map showing identified proteins divided into four clusters according to the expression patterns during postnatal development. Red, increased abundance in Fbln4SMKO aortas; green, decreased abundance in Fbln4SMKO aortas. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; p, phosphorylated; t, total.
To identify key molecules involved in the aneurysm development, we compared the protein profiles of control and Fbln4<sup>S-MKO</sup> aortas harvested at P1 (before aneurysms develop) to those harvested at P30 (when aneurysms have been established) by 2D-DIGE (15). Many proteins showed changes in abundance after P14 (Fig. 1C), and we subjected each protein spot with more than twofold difference in three independent experiments to mass spectrometric analysis. The identified proteins were classified into four clusters according to the temporal changes in their abundance (Fig. 1D, Table 1, and fig. S2A). The abundance of 21 proteins was increased (2- to 5.7-fold), and the abundance of 14 proteins was decreased (−2- to −6.9-fold) in Fbln4<sup>S-MKO</sup> aortas compared to control. Consistent with previous findings (9), we found that the abundance of Myh11 and Tagln (also known as SM22) was decreased at P30 (Fig. 1D). Other SMC proteins such as Ppp1r12a (MYPT1; myosin phosphatase light chain kinase) also showed decreased abundance in Fbln4<sup>S-MKO</sup> aortas compared to control. In contrast, the abundance of ECM-related molecules such as LOX (lysyl oxidase), Coll1a2 (collagen type I α2 chain), CTGF (connective tissue growth factor), and Htra1 (serine peptidase 1) was increased as the aneurysms expanded (fig. S2A). Gene ontology analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/) revealed that a substantial number of genes were involved in actin cytoskeleton organization, including Cald1 (caldesmon 1) and cofilins (fig. S2B). We further validated the changes in abundance at the mRNA level by qPCR analysis (fig. S3).

### Table 1. Identification of proteins 1 to 35 by Orbitrap Velos or Q Exactive mass spectrometer. CFPFP (Central Proteomics Facilities Pipeline) version 2.0.3 was used for database searching against the UniProt mouse database. MW, theoretical molecular weight (kD); pl, theoretical isoelectric point.

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein</th>
<th>Accession no.</th>
<th>Symbol</th>
<th>MW</th>
<th>pl</th>
<th>Peptides&lt;sup&gt;*&lt;/sup&gt;</th>
<th>% Coverage&lt;sup&gt;†&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Collagen type 1 α2 chain</td>
<td>NP_031769</td>
<td>Col1a2</td>
<td>129.6</td>
<td>9.3</td>
<td>7</td>
<td>5.9</td>
</tr>
<tr>
<td>2</td>
<td>Elongation factor 2</td>
<td>NP_031933</td>
<td>Eef2</td>
<td>95.3</td>
<td>6.4</td>
<td>14</td>
<td>14.0</td>
</tr>
<tr>
<td>3</td>
<td>Moein</td>
<td>NP_034963</td>
<td>Msn</td>
<td>66.5</td>
<td>5.9</td>
<td>51</td>
<td>70.9</td>
</tr>
<tr>
<td>4</td>
<td>Caldesmon 1</td>
<td>NP_663550</td>
<td>Cald1</td>
<td>60.5</td>
<td>7.0</td>
<td>46</td>
<td>89.2</td>
</tr>
<tr>
<td>5</td>
<td>Dihydropyrimidinase-like 3</td>
<td>NP_001129558</td>
<td>Dpps3</td>
<td>61.9</td>
<td>6.0</td>
<td>12</td>
<td>26.4</td>
</tr>
<tr>
<td>6</td>
<td>Serine protease HTRA1</td>
<td>NP_062510</td>
<td>Htra1</td>
<td>51.2</td>
<td>7.8</td>
<td>22</td>
<td>45.0</td>
</tr>
<tr>
<td>7</td>
<td>Serpin H1</td>
<td>NP_001104514</td>
<td>Serpinh1</td>
<td>46.5</td>
<td>8.9</td>
<td>13</td>
<td>30.0</td>
</tr>
<tr>
<td>8</td>
<td>Serine-threonine kinase receptor–associated protein</td>
<td>NP_035629</td>
<td>Strap</td>
<td>38.4</td>
<td>5.0</td>
<td>4</td>
<td>16.9</td>
</tr>
<tr>
<td>9</td>
<td>Tropomyosin 3</td>
<td>NP_071709</td>
<td>Tpm3</td>
<td>32.8</td>
<td>4.0</td>
<td>26</td>
<td>60.2</td>
</tr>
<tr>
<td>10</td>
<td>14-3-3 protein</td>
<td>NP_033562</td>
<td>Ywhea</td>
<td>29.2</td>
<td>4.6</td>
<td>28</td>
<td>74.9</td>
</tr>
<tr>
<td>11</td>
<td>Protein-lysine 6-oxidase</td>
<td>NP_034858</td>
<td>Lox</td>
<td>46.7</td>
<td>8.7</td>
<td>6</td>
<td>15.8</td>
</tr>
<tr>
<td>12</td>
<td>Annexin A2</td>
<td>NP_031611</td>
<td>Anxa2</td>
<td>38.6</td>
<td>7.6</td>
<td>5</td>
<td>32.4</td>
</tr>
<tr>
<td>13</td>
<td>Rho dissociation inhibitor 2</td>
<td>NP_031512</td>
<td>Arhgdib</td>
<td>22.9</td>
<td>5.0</td>
<td>13</td>
<td>57.0</td>
</tr>
<tr>
<td>14</td>
<td>Carbonic anhydrase 3</td>
<td>NP_031632</td>
<td>Car3</td>
<td>29.4</td>
<td>6.9</td>
<td>6</td>
<td>24.6</td>
</tr>
<tr>
<td>15</td>
<td>Peptidyl-prolyl cis-trans isomerase C</td>
<td>NP_032934</td>
<td>Ppic</td>
<td>22.8</td>
<td>7.0</td>
<td>2</td>
<td>11.8</td>
</tr>
<tr>
<td>16</td>
<td>Protein S100-A11</td>
<td>NP_058020</td>
<td>S100a11</td>
<td>11.0</td>
<td>5.3</td>
<td>6</td>
<td>51.0</td>
</tr>
<tr>
<td>17</td>
<td>Phosphodiesterase 4D, cAMP-specific (fragment)</td>
<td>NP_035186</td>
<td>Pde4d</td>
<td>84.5</td>
<td>4.8</td>
<td>2</td>
<td>2.3</td>
</tr>
<tr>
<td>18</td>
<td>Protein S100-A13</td>
<td>NP_033139</td>
<td>S100a13</td>
<td>17.7</td>
<td>6.2</td>
<td>5</td>
<td>43.9</td>
</tr>
<tr>
<td>19</td>
<td>Cytochrome c oxidase subunit 6B1</td>
<td>NP_079904</td>
<td>Cox6b1</td>
<td>10.1</td>
<td>9.0</td>
<td>2</td>
<td>11.6</td>
</tr>
<tr>
<td>20</td>
<td>Protein S100-A6</td>
<td>NP_035443</td>
<td>S100a6</td>
<td>10.1</td>
<td>5.3</td>
<td>2</td>
<td>16.9</td>
</tr>
<tr>
<td>21</td>
<td>Secem1</td>
<td>NP_081544</td>
<td>Scm1</td>
<td>46.3</td>
<td>4.7</td>
<td>7</td>
<td>15.2</td>
</tr>
<tr>
<td>22</td>
<td>Connective tissue growth factor</td>
<td>NP_034347</td>
<td>Ctfg</td>
<td>38.6</td>
<td>7.6</td>
<td>3</td>
<td>12.1</td>
</tr>
<tr>
<td>23</td>
<td>Transgelin (SM-22)</td>
<td>NP_035656</td>
<td>Tagln</td>
<td>22.5</td>
<td>8.9</td>
<td>17</td>
<td>63.2</td>
</tr>
<tr>
<td>24</td>
<td>Hemoglobin subunit p1</td>
<td>NP_032246</td>
<td>Hbb-b1</td>
<td>15.7</td>
<td>7.1</td>
<td>7</td>
<td>49.7</td>
</tr>
<tr>
<td>25</td>
<td>Myosin light polypeptide kinase</td>
<td>NP_647461</td>
<td>Mylk</td>
<td>213.6</td>
<td>5.9</td>
<td>14</td>
<td>15.2</td>
</tr>
<tr>
<td>26</td>
<td>Leucine zipper transcription factor–like 1</td>
<td>NP_021579</td>
<td>Lzfl1</td>
<td>34.7</td>
<td>5.1</td>
<td>6</td>
<td>21.5</td>
</tr>
<tr>
<td>27</td>
<td>Sepiapterin reductase</td>
<td>NP_035597</td>
<td>Spr</td>
<td>27.9</td>
<td>5.9</td>
<td>6</td>
<td>37.4</td>
</tr>
<tr>
<td>28</td>
<td>Heat shock protein p1</td>
<td>NP_038588</td>
<td>Hspb1</td>
<td>23.0</td>
<td>6.1</td>
<td>5</td>
<td>31.4</td>
</tr>
<tr>
<td>29</td>
<td>Myosin regulatory light polypeptide 9</td>
<td>NP_742116</td>
<td>Myl9</td>
<td>19.9</td>
<td>4.8</td>
<td>6</td>
<td>33.7</td>
</tr>
<tr>
<td>30</td>
<td>Cofilin-2</td>
<td>NP_031714</td>
<td>Clf2</td>
<td>18.7</td>
<td>7.7</td>
<td>9</td>
<td>51.8</td>
</tr>
<tr>
<td>31</td>
<td>Cofilin-1</td>
<td>NP_031713</td>
<td>Clf1</td>
<td>18.6</td>
<td>8.2</td>
<td>8</td>
<td>51.8</td>
</tr>
<tr>
<td>32</td>
<td>Prefoldin subunit 2</td>
<td>NP_035200</td>
<td>Pldn2</td>
<td>16.5</td>
<td>6.2</td>
<td>7</td>
<td>45.5</td>
</tr>
<tr>
<td>33</td>
<td>Fatty acid–binding protein, heart</td>
<td>NP_034304</td>
<td>Fabp3</td>
<td>14.9</td>
<td>6.1</td>
<td>8</td>
<td>54.9</td>
</tr>
<tr>
<td>34</td>
<td>Glutathione S-transferase μ</td>
<td>NP_034488</td>
<td>Gstm1</td>
<td>26.0</td>
<td>7.7</td>
<td>11</td>
<td>47.2</td>
</tr>
<tr>
<td>35</td>
<td>Mimecan (osteoglycin)</td>
<td>NP_032786</td>
<td>Ogn</td>
<td>34.0</td>
<td>5.5</td>
<td>5</td>
<td>13.8</td>
</tr>
</tbody>
</table>

*Number of matched peptides. †Percentage of sequence coverage.
extracellular face of the membrane to elastin and microfibril extensions radiating from the surface of the elastic laminae (18). Proper actin dynamics and orientation are therefore critical so that SMCs can generate and distribute appropriate forces to the elastic laminae and across the vessel wall. On the basis of these reasons, we focused on cofilins for further analysis. Cofilin-1 and cofilin-2 belong to the ADF/cofilin family of actin depolymerizing factors, which rapidly sever polymerized actin, thereby triggering the disassembly of actin fibers (19). In addition to direct regulation of actin remodeling, cofilin is also involved in various cellular functions of SMCs, including platelet-derived growth factor (PDGF)-induced migration and neoinstima formation (20, 21). qPCR analysis showed that the abundance of the mRNA encoding cofilin-2 (muscle cofilin) was decreased at P14 and P30, whereas that of the mRNA encoding cofilin-1 (nonmuscle cofilin) was unchanged until P14 and then markedly increased at P30 (fig. S3). Cofilin is inactivated when phosphorylated by LIM kinases (LIMKs), thereby leading to decreased actin binding, severing, and depolymerization (22). Western blotting revealed that the phosphorylation of cofilin started to decrease at P7 and was significantly decreased at P14 in Fbln4SMKO aortas, which coincided with thickening of the medial layers. Total cofilin abundance was similar in both genotypes (Fig. 2A). Consistently, immunostaining revealed that the phosphorylation of cofilin was markedly decreased in P30 Fbln4SMKO ascending aortas and, to a lesser extent, in the descending aorta (Fig. 2B and fig. S4). These data indicated that cofilin activity was significantly increased by either compromised phosphorylation or accelerated dephosphorylation.

Because LIMKs are regulated by RhoA signaling, we examined whether RhoA abundance was decreased in Fbln4SMKO aorta. Instead, Western blot analysis showed that total RhoA abundance began to increase in Fbln4SMKO ascending aortas starting at P7 (fig. S5A) and that active RhoA was also increased in Fbln4SMKO aortas (fig. S5B). Accordingly, phosphorylation of downstream molecules, including myosin light chain (MLC), myosin-binding subunit of myosin phosphatase (MYPT), myosin light chain kinase (MLCK), and LIMK, was also substantially increased in Fbln4SMKO aortas (fig. S5, C and D), indicating that RhoA-LIMK signaling was activated in the mutant aorta, consistent with the increase in AngII signaling in the mutant aortic wall (14).

Several phosphatases, including the SSH family (composed of SSH1, SSH2, and SSH3 in mammals) and CIN, can dephosphorylate cofilin and restore its activity (23, 24). We therefore examined if cofilin phosphatases were dysregulated in Fbln4SMKO aortas. The abundance of SSH1, but not that of SSH2, SSH3, or CIN, was significantly increased at P7 in Fbln4SMKO aortas, preceding the decrease in the phosphorylation of cofilin (Fig. 2A). These data suggested that cofilin was dephosphorylated by SSH1 in the vessel wall and that cofilin activity was increased in Fbln4SMKO aortas. Indeed, when overexpressed in rat vascular SMCs, wild-type SSH1, but not phosphatase-inactive SSH1 (SSH1-CS), dephosphorylated cofilin, confirming its phosphatase activity as previously described (fig. S6, A and B) (25). To further confirm these findings, we examined monomeric actin (G-actin) and filamentous actin (F-actin) (Fig. 2C). The ratio of G-actin to F-actin was significantly increased in ascending aortic extracts from Fbln4SMKO mice compared to control mice, whereas descending aortic extracts did not show statistically significant differences between genotypes (Fig. 2C). Accordingly, actin distribution was disrupted in the P30 Fbln4SMKO aortas compared to control aortas (Fig. 2D). These data suggested that increased cofilin activity led to an increase of G-actin and affected actin filament integrity.
Fig. 3. Disruption of elastic lamina–SMC connections and alteration of the mechanical properties of Fbln4SMKO aortas. (A) Electron microscopy images from CTRL and Fbln4SMKO (SMKO) ascending aortas at P90 and P7. Elastic lamina (EL)–SMC connections are well formed in CTRL aortas (white arrows), whereas elastic laminae are disrupted and not connected to SMCs in the Fbln4SMKO aorta at P90. Elastic laminae were also abnormal at P7 in Fbln4SMKO vessels, with numerous globules of elastin rather than solid bands of elastin (white arrowhead) and less organized cell-elastin associations (white arrows). Scale bars, 1 μm. Images are representative of at least \( n = 2 \) (CTRL) and \( n = 3 \) (Fbln4SMKO) mice per age. (B) Upper panel: Aortic pressure–outer diameter curves for P1, P7, P14, and P30 ascending aorta. Fbln4SMKO aortas at P14 have significantly large outer diameter than CTRL. Lower panel: Aortic pressure–compliance curves for CTRL and Fbln4SMKO ascending aortas. Fbln4SMKO aortas show significant differences beginning at P7. \( n = 5 \) to 8 mice per group. Bars are means ± SD. \(* P < 0.05\), \(** P < 0.01\), \(*** P < 0.001\), generalized estimating equation. (C) Western blots showing the abundance of ACE, TSP1 (thrombospondin-1), Egr1 (early growth response 1), and the phosphorylation of ERK are increased by transverse aortic constriction (TAC) in wild-type mice. \( n = 4 \) mice for sham and \( n = 5 \) mice for TAC. Bars are means ± SEM. \(* P < 0.05\), \(*** P < 0.001\), \(**** P < 0.0001\). Exact Wilcoxon rank sum test for comparison with TSP1 in sham group. All the other comparisons were done by unpaired \( t \) test. (D) Western blots showing Egr1 and TSP1 abundance is increased in postnatal Fbln4SMKO aortas. The experiment was performed three times with different pools of animals with similar results. \( n \) values as in Fig. 1A. \(* P < 0.05\), \(** P < 0.01\), \(*** P < 0.001\), unpaired \( t \) test.
which could potentially contribute to the development of aneurysms in Fbln4^−/− mice.

**Elastic lamina–SMC connections were disrupted and mechanical properties were altered in Fbln4^−/− aortas**

The activation of cofilin and expansion of aneurysms prompted us to investigate the trigger for these changes. Cofilin can serve as a cellular mechanosensor in an in vitro reconstitution system (26). Therefore, we hypothesized that cofilin activation was induced as a result of abnormal mechanosensing and/or mechanotransduction in Fbln4^−/− SMCs. To test this hypothesis, we first examined the ultrastructure of the established aneurysmal wall at P90. Extensive connections were present between the elastic laminae and SMCs in control aortas (indicated by white arrows in Fig. 3A, CTRL, P90) but not in Fbln4^−/− aortas, which had large, irregular aggregates of elastin with a lacy appearance between SMCs instead of continuous elastic laminae (Fig. 3A, SMKO, P90). We next examined if similar changes were apparent in P7 aortas. Wild-type P7 aortas had near-complete elastic laminae and early connections between the elastic laminae and SMCs, although they were not as fully established as those in adult aortas (Fig. 3A, CTRL, P90, white arrows). In the Fbln4^−/− aortas, the elastic laminae appeared to be fragmented into juxtaposed, individual elastin globules (Fig. 3A, SMKO, P7, arrowhead). In some areas, connections appeared to be disrupted because of the abnormal organization of the elastic laminae (Fig. 3A, SMKO, P7, white arrows).

We examined temporal changes in the biomechanical properties of the aortas by pressure-diameter analysis (Fig. 3B). The outer diameter was similar between the genotypes at P1 or P7; however, aneurysmal wall changes were detected at P14 (Fig. 3B, upper panel). Compliance, which is the inverse of stiffness, was significantly different between the genotypes from P7, and it increased at low pressures and decreased at or above physiological pressures in the mutants from P7 (Fig. 3B, lower panel).

**The abundance of Egr1 was increased in Fbln4^−/− aortas**

We wondered how mechanical alteration and loss of elastic laminae–SMC connections were converted to intracellular signaling. Integrins and focal adhesion kinase (FAK) are mechanosensors that are found in the membrane-associated dense plaques of SMCs (27). The phosphorylation of FAK was transiently decreased in Fbln4^−/− aortas at P1, but total abundance did not differ between the genotypes after P7 (fig. S7A). The phosphorylation of integrin-linked kinase (ILK) was similar between control and Fbln4^−/− aortas at all time points (fig. S7, A and B). Egr1 is a zinc finger transcription factor that is induced by AngII in cardiac fibroblasts and by mechanical stress in vascular SMCs (28, 29) and that increases the abundance of TSP1 and ACE (30, 31). We therefore examined if changes in the abundance of these molecules correlated with changes in mechanical stress in the aortic wall. TAC increases mechanical force and lead to aortic dilatation and moderate thickening of the medial layers, which is associated with an increase in the phosphorylation of ERK (32). On the basis of this finding, we tested if Egr1 could respond to mechanical stress applied to the ascending aortic wall by TAC in wild-type mice (33). Western blot analysis showed a significant increase in the abundance of Egr1, ACE, and TSP1 in TAC-operated ascending aortas (Fig. 3C). These data suggested that Egr1, ACE, and TSP1 are mechanical stress–responsive genes in vivo. The phosphorylation of cofilin was also decreased slightly in TAC-operated animals compared to sham-operated animals (Fig. 3C). We next examined if mechanosensing of SMCs was altered in Fbln4^−/− aortas. The abundance of Egr1 was transiently increased at P1 and remained high in Fbln4^−/− aortas from P14, whereas that of TSP1 was significantly increased at P30, which was maintained until P90 (Fig. 3D). These results indicated that Fbln4^−/− aortas showed biochemical changes similar to pressure overload even under the normal blood pressure conditions that are present in 6- to 8-week-old mutant mice (14).

**Postnatal Fbln4 deletion did not lead to cytoskeletal changes and aneurysm formation in vivo**

To confirm if structural defects involving the association of the SMCs with the elastic laminae contributed to the full development of the aneurysmal phenotype in vivo, we generated tamoxifen-inducible Fbln4 knockout mice by using SMA-Cre-ER^T2^, which induces Cre-recombinase in...
vascular SMCs [inducible SMKO (iSMKO); Fbln4<sup>−/−</sup>;SMA-Cre-ER<sup>T2</sup>]. Mice were injected with tamoxifen for five consecutive days beginning at P7, a time point after which the elastic laminae have already been essentially assembled in the aortic wall (35). Tamoxifen-injected Fbln4<sup>+/−;Cre<sup>−</sup></sup> or Fbln4<sup>−/−</sup>;Cre<sup>+</sup> mice served as controls. In both control and iSMKO mice, aortas were normal and did not have dilatations or aneurysms (Fig. 4A). In addition, the descending aorta did not exhibit a tortuous appearance, which is a characteristic of most mouse models with defective elastic laminae assembly (such as Fbln5<sup>−/−</sup> and Eln<sup>−/−</sup> mice) (36, 37). Deletion of Fbln4 was confirmed by qPCR (Fig. 4B). To examine whether molecular changes had occurred in the iSMKO aorta, we examined the abundance of mechanosensitive molecules as well as the phosphorylation of ERK1/2 and cofilin by Western blot analysis (Fig. 4C). Consistent with a normal phenotype, biochemical markers were similar between control and iSMKO aortas. Immunostaining of cross sections of the iSMKO and control aortas also showed intact elastic laminae and robust staining for phosphorylated cofilin (Fig. 4D). These data suggest that deletion of Fbln4 after the establishment of elastic lamina–SMC connections does not cause abnormal mechanosensing in SMCs under baseline conditions.

**Losartan effectively decreased SSH1 and cofilin, preventing aneurysm formation**

We have previously shown that losartan reverses both the increase in the phosphorylation of ERK and the reduction of the expression of SMC differentiation markers in Fbln4<sup>SMKO</sup> aortas; we have also established a critical therapeutic time window (P7 to P30) for preventing aneurysm development (14). To examine whether Egr1, SSH1, and cofilin were associated with the therapeutic effects of losartan, we compared their abundance at P30 in aortas from Fbln4<sup>SMKO</sup> mice treated or not with losartan between P7 and P30. Losartan treatment markedly decreased the phosphorylation of ERK, partially prevented the increase in the abundance of Egr1 and SSH1, and partially restored the phosphorylation of cofilin (Fig. 5A). Consistently, losartan increased the phosphorylation of cofilin and improved the morphology of elastic fibers in Fbln4<sup>SMKO</sup> aortas to that detected in control vessels (Fig. 5B), and phalloidin staining showed Fig. 5. The effects of losartan on cofilin activity and aneurysm formation. (A) Effects of losartan (LSRT) treatment from P7 to P30 on ACE, TSP1, Egr1, phosphorylated ERK, total ERK, phosphorylated cofilin, cofilin, and SSH1 abundance in Fbln4<sup>SMKO</sup> (SMKO) aortas (pooled three to six aortas per sample; 9 to 17 mice per genotype and treatment). All animals were evaluated at P30. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, one-way analysis of variance (ANOVA). (B and C) Cross sections of the ascending aorta from Fbln4<sup>SMKO</sup> with losartan treatment from P7 to P30. n = 5 mice per genotype and treatment. Scale bars, 50 μm. (B) Immunostained with phosphorylated cofilin (red) and DAPI (blue). Elastic laminae are green (autofluorescence). (C) Immunostained with phalloidin (red) and DAPI (blue). n = 5 mice per treatment. (D) Effects of postnatal losartan treatment on aneurysm formation. All animals were evaluated at P90. n = 3 mice per genotype and treatment. *P < 0.05, **P < 0.01, ****P < 0.0001, one-way ANOVA.
well-organized actin assembly (Fig. 5C, compare to the phalloidin staining in Fig. 2D). These data suggested that losartan targeted mechanosensitive molecules downstream of AT1R between P7 and P30.

To further examine the relationship between losartan and downstream signaling pathways, we used two losartan treatment protocols in which we treated animals from P7 to P45 or P45 to P90 and harvested aortas from both groups at P90 (Fig. 5D), as previously described (14). Consistent with a previous report (14), aneurysms were completely prevented in Fbln4SMKO mice receiving losartan during the early postnatal period, even when losartan was withdrawn from P45 to P90 (LSRT P7–P45). Treatment starting at P45 did not rescue aneurysm formation under continuous administration of losartan up to P90 (LSRT P45–P90) (Fig. 5D). Surprisingly, the phosphorylation of ERK was increased in Fbln4SMKO aortas after losartan withdrawal. The abundance of Egr1 and SSH1 was decreased only in aortas from Fbln4SMKO mice, which had been treated in the early postnatal period (P7 to P45), and the phosphorylation of cofilin was increased to an amount comparable to that detected in control mice. Together, abnormal mechanosensing and activation of AngII-AT1R pathway led to the activation of cofilin through SSH1 between P7 and P30, which is consistent with the therapeutic window of losartan in Fbln4SMKO aortas.

**Inhibition of the SSH1-cofilin pathway prevents aortic aneurysms in vivo**

We finally asked if we could prevent aneurysm formation by pharmacologically inhibiting the SSH1-cofilin pathway. Phosphoinositide 3-kinase (PI3K) is involved in insulin- or AngII-induced activation of SSH1 in vitro (38–40). Therefore, we treated Fbln4SMKO mice with the PI3K inhibitors wortmannin or LY294002 from P7 to P30 and examined the formation of aneurysms at P30. Neither drug affected body weight at P30 (Fig. S8A). Although wortmannin or LY294002 treatment successfully ameliorated aneurysm formation, tortuosity of the descending aorta was still evident (Fig. 6A and figs. S8B and S9A). In addition, aortas from these drug-treated Fbln4SMKO mice had smaller lumens compared to those from untreated mutants but have thicker aortic walls compared to those of control animals, suggesting a balancing of wall tension by an increase in the thickness of aortic wall (Fig. 6B and fig. S9B). Biochemically, both inhibitors decreased the phosphorylation of AKT as well as that of ERK1/2 (Fig. 6C and fig. S9C), and the abundance of TSP1 and Egr1 was decreased in PI3K inhibitor–treated Fbln4SMKO aortas. As expected, SSH1 abundance was significantly decreased and the phosphorylation of cofilin was increased to the amount seen in control mice.

---

**Fig. 6. The involvement of PI3K in aortic aneurysm formation.** (A) Gross photos of CTRL and Fbln4SMKO (SMKO) aortas with or without wortmannin treatment. Arrow shows a tortuous descending aorta. Images are representative of 11 to 16 mice per genotype. (B) Histological images of cross sections of the ascending aorta from wortmannin (HWT)-treated CTRL, Fbln4SMKO, and untreated Fbln4SMKO mice stained with hematoxylin and eosin (H&E), Hart’s (elastin), and Masson trichrome (collagen). Scale bars, 500 μm (×5) and 20 μm. Images are representative of four mice per genotype. (C) Western blots showing the effect of wortmannin treatment (pooled three aortas per sample) compared to vehicle-treated Fbln4SMKO (pooled two aortas per sample). Six to nine mice per genotype and treatment. *P < 0.05, **P < 0.01, ****P < 0.0001, one-way ANOVA.
Several upstream signaling pathways regulate the phosphatase activity of SSH1 (41). Protein kinase D (PKD) inhibits SSH1 activity by phosphorylating SSH1 and inducing its binding to the scaffold protein 14–3–3. Conversely, calcium-induced signaling induces SSH1 activation through the calcineurin-mediated dephosphorylation of SSH1 (45). In Fbln4\textsuperscript{SMKO} aortas, the phosphorylation of PKD was unchanged compared to control aortas (fig. S10A), and activation of calcineurin as deduced by the abundance of its inhibitor RCAN1.4 remained increased even after inhibition of wortmannin or LY294002 (fig. S10, B to D). Together, these results suggested that cofilin is downstream of activation of the AT1R by AngII and regulated by PI3K-dependent increases in SSH1 abundance, and appears to be involved in aneurysm progression in Fbln4\textsuperscript{SMKO} mice.

**DISCUSSION**

Using a combination of 2-D DIGE and established mouse models of ascending aortic aneurysms, we investigated the molecular mechanism(s) driving aneurysm initiation and expansion. We showed that structural changes involving elastic lamina–SMC connections and alteration in mechanical properties of the aortic wall were associated with abnormal mechanosensing of SMCs as evidenced by increasing the abundance of mechanosensitive molecules Egr1, ACE, and TSP1. Subsequent activation of the AngII-AT1R pathway led to aneurysm formation in a manner that is dependent on PI3K and the SSH1-cofilin pathway (Fig. 7).

**Aneurysm initiation was triggered by altered mechanosensing of Fbln4\textsuperscript{SMKO} SMCs**

Temporal expression analysis of Fbln4\textsuperscript{SMKO} aortas before the initiation of aneurysmal changes revealed that differentiated Fbln4\textsuperscript{SMKO} SMCs underwent progressive phenotypic changes from P7 to P30. This period coincides with the completion of elastic laminae formation and the development of mature elastic lamina–cell associations (18, 35), as well as an increase in postnatal blood pressure under physiological conditions (46). In normal development of the aortic wall, fibrillin-1 microfibrils provide a scaffold for elastic lamina formation. From late embryogenesis, and during the few weeks of postnatal life, elastin is continually deposited onto this scaffold until mature elastic lamina are formed (18, 35). The fibrillin-1 microfibrils also form connections between the elastic laminae and the SMC surface, which become increasingly infiltrated with elastin, anchoring on the cell membrane in regions occupied intracellularly by electron-dense plaques. Because the dense plaques are the sites of actin filament attachment, a continuum of tension is established from within the cell to the elastic laminae (35). Fbln4\textsuperscript{SMKO} aortas exhibited altered biomechanical properties and distinct biochemical responses compared to wild-type aortas with an increase in the abundance of mechanosensitive molecules Egr1, ACE, and TSP1. These observations suggest that tethering of SMCs to the elastic laminae is required for the proper distribution of increasing wall stress and homeostasis of cellular tension as well as maintenance of the differentiated status of SMCs. Loss of appropriate elastic lamina–SMC connections is instrumental for development of aneurysms, because neither deletion of Fbln4 in SMCs after the connections are formed nor induction of mechanosensitive genes alone without structural defects in wild-type aorta by TAC was sufficient to induce ascending aortic aneurysms in vivo.

Mice with reduced expression of fibrillin-1 (Fbn1\textsuperscript{mgR/mgR}) develop dilated cardiomyopathy due to impaired mechanotransduction of cardiac myocytes, which involves integrin β1 and FAK (47). Fbn1\textsuperscript{mgR/mgR} cardiac myocytes exhibited nearly absent phosphorylation of FAK and increased phosphorylation of ERK1/2 and Smad2/3, effects that were rescued by losartan but not by an ACE inhibitor or deletion of angiotensinogen. Because fibrillin-1 has an RGD domain that binds the cell surface integrin β1 (48), it was suggested that a primary defect was a loss of mechanotransduction mediated by fibrillin-1–cell interactions. Here, loss of fibrillin-4 has no known cell surface receptors and because it potentiates binding between LOX and tropoelastin in vitro (49), it is likely that fibrillin-4 is involved in deposition of elastin and formation of elastic lamina–SMC connections, thereby indirectly regulating mechanotransduction mediated by cell–elastic lamina connections. Together, our findings support the involvement of altered mechanotransduction in development of aortic aneurysms in humans (50).

**A feed-forward loop involving ACE and AngII leads to abnormal actin remodeling and aneurysm expansion**

The earliest and consistent biochemical change in Fbln4\textsuperscript{SMKO} aortas was the increased abundance of Egr1, followed by those of ACE and TSP1. Because Egr1 is a transcriptional activator of ACE and downstream target of AngII (29), it is likely that abnormal mechanosensing by SMCs is biochemically translated as the increased abundance of Egr1 followed by local increase of ACE, resulting in production of AngII and activation of the AngII–AT1R pathway. Our analysis also suggests that cofilin is downstream of the AngII–AT1R pathway and regulated by PI3K. The possibility that SSH1 is directly induced by Egr1 and activating cofilin needs to be tested. Our observation that substantial mechanical and biochemical changes occur at P7 in Fbln4\textsuperscript{SMKO} mice emphasizes the importance of a therapeutic
time window that targets the establishment of proper connections between elastic lamina and SMCs for effective prevention of aortic aneurysms.

Actin filament remodeling by cofilin is regulated by the balance between phosphorylation and dephosphorylation through LIMK and cofilin phosphatases, respectively (57). Although the abundance of RhoA and LIMK was increased in the Fbln4 knockout (Fbln4/−/−) aorta, which would be expected to enhance F-actin formation, the increase in actin polymerization appeared to be negated by increased cofilin activity. The reduced F-actin potentially affects aneurysm expansion by inhibiting myocardin-related transcription factor A (MRTF-A)-dependent, CArG box-containing, SMC contractile gene expression such as Acta2, Myh11, and Cnn1, and decreasing the contractile forces of SMCs, as has been shown in mice carrying a mutation in MYH11p<sub>2247C</sub> (52, 53). Cofilin also increases mitochondria size and abundance through actin remodeling in a serum response factor (SRF)-dependent manner (54). Active cofilin translocates to mitochondria together with BAX and induces apoptosis in cardiac myocytes independently of actin filaments (55). These observations indicate that activated cofilin exerts multiple cellular functions in both actin-dependent and actin-independent manner. The exact role of cofilin during aneurysm development, therefore, warrants further investigation.

Limitations and future prospective
Our study showed that the reduction in the abundance of SSH1 by PI3K inhibitors suppressed cofilin activity and prevented aortic aneurysms in Fbln4 knockout (Fbln4/−/−) aortas. These results may offer a new potential strategy to treat ascending aortic aneurysms and lumen enlargement in humans. Clinical trials comparing the effects of losartan to β-blocker atenolol on aneurysm growth in Marfan patients show that losartan has no advantages over atenolol (56). Although the underlying disease mechanism is different between Marfan syndrome and fibrillin-4 deficiency, it may be beneficial to target a specific intracellular signaling pathway downstream of AngII. In addition, the timing of administration of losartan should be carefully considered to exert most efficient therapeutic effects. Our current studies fail to directly measure the SSH1 activity during aneurysm formation. More thorough studies to determine the specificity of the SSH-cofilin pathway in ascending aortic aneurysms will be necessary to establish drug regimens for precisely controlling and preventing aneurysm formation.

MATERIALS AND METHODS

Mice
Fbln4 knockout (Fbln4/−/−) mice were generated as described previously (9, 14). Fbln4<sup>+/−</sup>, Fbln4<sup>+/+</sup>, or Fbln4<sup>−/−</sup> mice containing SM22α-Cre (57) transgene were used as controls in this study. All mice were kept on a 12-hour light/12-hour/dark cycle under specific pathogen-free condition, and all animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas (UT) Southwestern Medical Center.

Western blot analysis
Aortas were harvested and perivascular adipose tissue was thoroughly removed. Aortas were divided into ascending thoracic (from the aortic root to immediately past the left subclavian artery) and descending thoracic aortas, then minced in liquid nitrogen by pestle, and dissolved in radioimmunoprecipitation assay lysis buffer containing 1% protease inhibitor and 1% phosphate inhibitor (all from Sigma). The lysates were mixed with 3× SDS sample buffer with 2-mercaptoethanol, boiled at 95°C for 5 min, and then subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a polyvinylidene difluoride membrane (Millipore) and immunoblotted with antibodies indicated in table S1. Membranes were then incubated with respective anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:1000; Bio-Rad) and visualized with chemiluminescence kit (Santa Cruz Biotechnology) or with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific). Quantification of proteins normalized to GAPDH is shown in the graphs adjacent to Western blots, and bars are means ± SEM.

Quantitative PCR
RNA was isolated from aortas at the indicated age using RNaseasy Plus Micro Kit (Qiagen), and 1 μg of total RNA was subjected to reverse transcription reactions using iScript Reverse Transcription Supermix (Bio-Rad). SYBR Green was used for amplicon detection, and gene expression was normalized to the expression of housekeeping genes β2-microglobulin and GAPDH. PCRs were carried out in triplicate in a CFX96 real-time PCR detection system (Bio-Rad) with 1 cycle of 3 min at 95°C and then 39 cycles of 10 s at 95°C and 30 s at 55°C. mRNA abundance was determined using the delta ΔC<sub>t</sub> method and expressed relative to the mean ΔC<sub>t</sub> of controls. Primer sequences are provided in table S2.

Two-dimensional differential gel electrophoresis
Entire aortas from P1 (n = 4 mice) and ascending aortas from P7 (n = 7 mice), P14 (n = 5 mice), and P30 (n = 3 mice) per genotype (controls and Fbln4 knockout [Fbln4/−/−]) were collected and combined per each time point and prepared for three independent experiments. Aortas were homogenized in lysis buffer containing 7 M urea, 2 M thiourea, 3% (w/v) CHAPS, and 1% Triton X-100. Fifty micrograms of individual sample group was labeled with Cy3 or Cy5, and equal amount of sample mix was labeled with Cy2 as the internal standard (15). Labeled samples were mixed and applied to the immobilized pI gradient (IPG) strips (pH 3 to 10; GE Healthcare Life Sciences), and first-dimension isoelectric focusing was performed using an Ettan IPGphor 3 (GE Healthcare Life Sciences). The strips were mounted on top of the 12.5% SDS–polyacrylamide gel for second-dimensional protein separation by using an Ettan DALTSix system (GE Healthcare Life Sciences). After SDS-PAGE, gels were scanned using a Typhoon Variable Mode Imager (GE Healthcare Life Sciences) and quantified using the DeCyder version 6.5 software (GE Healthcare Life Sciences).

Protein identification
For spot picking, 500 μg of protein samples without fluorescent labeling was subjected to 2D-DIGE, and the gel was stained with DeepPurple (GE Healthcare Life Sciences). Protein spots of interest were picked with an Ettan Spot Picker (GE Healthcare Life Sciences). These spots were then destained, reduced, and alkylated, after which overnight digestion with trypsin was performed. The resulting tryptic peptides were extracted, dried, and cleaned by a SpeedVac. The peptides were then dried again and reconstituted in 10 μl of water, 0.1% trifluoroacetic acid. Samples were analyzed using either an Orbitrap Velos or Q Exactive mass spectrometer (Thermo Fisher) coupled with identical UltimaMate 3000 RSLCnano HPLC systems ( Dionex). Samples were loaded onto 75-μm inside diameter × 15 cm column and separated with a linear gradient from 0 to 40% buffer B, where buffer A is water with 0.1% formic acid (FA) and buffer B is acetonitrile with 0.1% FA. C18 column 2.03 was used for database searching against the UniProt mouse database (58, 59). All raw mass spectrometry data files have been deposited to the Mass spectrometry Interactive Virtual Environment (MassIVE; Center for Computational Mass Spectrometry at the University of California, San Diego) and can be accessed using the MassIVE ID MSV000079171. These raw data also link to the ProteomeXchange consortium (http://proteomexchange.org/) with the identifier PXD002423. Table S3 contains the list of accession file names of raw mass spectrometry data.
Hierarchical cluster analysis
Hierarchical cluster analyses were carried out using the Multiple Experimental Viewer (www.tm4.org/mev/). The proteins in a given cluster exhibited similar changes in abundance in a synergistic manner.

Immunostaining
Aortas were harvested and embedded in optimal cutting temperature (OCT) compound (Sakura Finetek USA Inc.) and snap-frozen in liquid nitrogen. Cross sections of the mouse aorta were immediately fixed with 4% paraformaldehyde for 30 min, blocked in 5% normal serum in which secondary antibody was raised, containing 0.1% Triton X for 1 h at 37°C, and then incubated with primary antibodies at the following concentrations (table S1). Incubations were performed overnight at 4°C. After washing, high cross-absorbed Alexa Fluor 568–conjugated secondary antibodies (Invitrogen) were added at a dilution of 1:200 for 2 h at 37°C. Slides were covered with Vectashield containing DAPI (Vector Laboratories) and viewed under an LSM 510 (Zeiss) or Axio Observer (Zeiss).

G- actin/F-actin assay
The G-actin/F-actin ratio was analyzed in control and Fbln4 SMKO aortas using a commercially available kit (Cytoskeleton Inc.) according to the manufacturer’s instructions. Briefly, harvested aortas were separated by ascending and descending regions and lysed with lysis buffer (kit provided) containing 1 mM adenosine triphosphate (ATP) to stabilize F-actin. The lysates were centrifuged at 100,000g for 1 h at 37°C using Beckman ultracentrifuge. The G-actin–containing supernatants were then separated from the F-actin–containing pellets. The pellets were resuspended to the same volume as the supernatants, using ice-cold water containing 10 μM cytochalasin D (kit provided). Equivalent volumes of corresponding F- and G-actin fractions were loaded onto an SDS-PAGE gel and analyzed by Western blot with anti–pan-actin antibody (kit provided). G- and F-actin were quantified by using ImageJ software [National Institutes of Health (NIH) Image].

RhoA activity assay
RhoA was assayed by a pull-down assay according to the manufacturer’s instructions for a RhoA Activation Assay Biochem Kit (Cytoskeleton). Briefly, aortas from control and Fbln4 SMKO mice were lysed in the lysis buffer provided in the kit. Guanosine triphosphate (GTP)–bound RhoA was then immunoprecipitated from cleared lysate with glutathione S-transferase–tagged Rhotekin Rho-binding domain protein bound to glutathione agarose. The beads were washed, and the immunoprecipitates were analyzed by Western blot using RhoA-specific monoclonal antibody provided in the kit. Blots were quantified by using ImageJ software (NIH Image).

Cell culture, transfection, and immunostaining
Rat vascular SMCs (Lonza) were maintained in Dulbecco’s modified Eagle’s medium/F12 supplemented with 20% fetal bovine serum and 0.1% GA-1000, containing gentamicin (30 μg/ml) and amphotericin (15 ng/ml) (Lonza). For immunostaining, cells were grown on four-well chamber slides (Thermo Fisher), and the plasmid DNA encoding human SSH-1L mRNA plasmid (gift from K. Mizuno) was transfected into cells using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions. Forty-eight to 72 h after transfection, cells were fixed in 100% methanol at −20°C for 20 min, blocked with 2% bovine serum albumin fraction V (Wako Chemical) containing 0.1% Tween 20 for 1 h, and incubated with phospho-cofilin antibodies (1:500; Santa Cruz Biotechnology) overnight at 4°C. Alexa Fluor 568–conjugated secondary antibodies (Invitrogen) were used at a dilution of 1:200 for 1 h at 37°C. Slides were covered with Vectashield containing DAPI (Vector Laboratories). Fluorescence images were obtained using an LSM700 (Zeiss).

Electron microscopy
Aortas were dissected from P7 and P90 mice after cardiac perfusion with 3% glutaraldehyde in 0.1 M sodium cacodylate, and samples were prepared as previously described (9). Sections were viewed using a Tecnai 12 transmission electron microscope at 120 kV, and images were digitally captured. At least two wild-type and three mutant animals at each time point per genotype were used.

Transverse aortic constriction
Eleven-week-old male C57BL/6 mice underwent TAC using a standard surgical protocol (33) with modifications. Briefly, anesthetized mice were placed in a spine position, and aortic constriction was achieved by tying a 7-0 polypropylene suture against a 27-gauge blunt needle. For the sham group, the same operation was performed without ligating the aorta. Studies were carried out 3 weeks after TAC, before the maladaptive cardiac failure associated with TAC.

Generation of inducible SMC-specific fibulin-4 knockout mice
Transgenic mice have been previously described (34). Fbln4 SMKO mice carrying the SMA-Cre-ER T2 transgene were designated as iSMKO. Mice were injected with tamoxifen (0.1 mg/g) for 5 consecutive days beginning at P7, and then aortas were harvested at P60 for detection of aortic aneurysms. Deletion of Fbln4 was confirmed by qPCR using aortas harvested at P60. The primers used are in table S2.

Losartan experiment
Losartan [0.6 g/liter in drinking water, ad libitum, provided by Merck and Co. Inc. under material transfer agreement (MTA) 38648] was administered during P7 to P30 or P45 and P45 to P90 as previously described (14).

Wortmannin or LY294002 treatment in vivo
Fbln4 SMKO and control pups were divided into two groups; vehicle control and inhibitor treatment. Pups were injected intraperitoneally with 17β-hydroxy analog wortmannin (0.25 mg/kg; Calbiochem), LY294002 (3.0 mg/kg; Calbiochem), or phosphate-buffered saline (containing 0.05% dimethyl sulfoxide) as previously reported (60–62). The treatments were continued everyday from P7 to P14 and then every other day from P14 to P30. At P30, pups were sacrificed and aortas were harvested and evaluated for aneurysm phenotypes.

Histology and morphometric analysis
Aortas were harvested and perfusion-fixed with 4% paraformaldehyde and embedded in paraffin. Five-micrometer sections were stained with H&E for routine histology and Masson’s trichrome staining for detection of collagen fibers. Cross sections of the aorta were stained with Hart’s stain to visualize elastic fibers, and images were digitally captured with Leica DM2000 microscope. Morphometric analysis was performed with NIH ImageJ software as described previously (14).

Mechanical testing
Mechanical testing was performed as previously described (63). Briefly, each aorta was horizontally mounted in the mechanical test device (Myograph 110P; DMT) in physiologic saline and stretched in the longitudinal direction to the approximate in vivo length and then was pressurized from 0 mmHg (5 mmHg for PI), because the wall was often collapsed at 0 mmHg) to two to three times the physiological blood pressure of the mouse (PI,
60 mmHg; P7, 90 mmHg; P14, 130 mmHg; and P30, 175 mmHg). The vessel was preconditioned before obtaining the experimental data, and changes in the outer diameter, internal pressure, and axial force were recorded for analysis. The mechanical testing was completed within 3 days of dissection.

Statistical analysis
All experiments are presented as means ± SEM except Fig. 3B, where SD was used. Kolmogorov-Smirnov tests were conducted to examine whether the data followed normal distribution. If the data followed a normal distribution, statistical significance was determined by unpaired t test for two-group comparisons and one-way ANOVA for comparison among three or more groups followed by Bonferroni’s correction for multiple comparison tests. If the normality assumption was violated, nonparametric tests were conducted. Exact Wilcoxon rank sum tests were used for two-group comparison (Figs. 3C and 4C), and Kruskal-Wallis tests were used for comparisons among three or more groups followed by Bonferroni’s correction for multiple comparison tests (fig. S8B). Generalized estimating equation approach was used for the analysis of repeated measurement data (Fig. 3B). P < 0.05 denotes statistical significance.

SUPPLEMENTARY MATERIALS
www.sciencesignaling.org/cgi/content/full/8/399/ra105/DC1
Fig. S1. ACE abundance and ERK phosphorylation in Fbln4SMKO aortas after P7.
Fig. S2. Ontology analysis and proteins that showed changes in abundance during aneurysm development.
Fig. S3. qPCR analysis of genes encoding the proteins identified by 2D-DIGE.
Fig. S4. Phosphorylation of collagen in Fbln4SMKO ascending aortas.
Fig. S5. Activated RhoA signaling in Fbln4SMKO ascending aortas.
Fig. S6. Dephosphorylation of collagen by SSH1 in rat vascular SMCs.
Fig. S7. Phosphorylation of FAK and ILK in ascending aortas.
Fig. S8. The effects of P38K inhibitors on general growth and aneurysm development.
Fig. S9. The effect of LY294002 on aneurysm development in Fbln4SMKO mice.
Fig. S10. Evaluation of PKD phosphorylation and calcineurin activity in ascending aortas.
Table S1. Antibodies used in this study.
Table S2. qPCR primer sequences.
Table S3. List of links to raw data for mass spectrometry.

REFERENCES AND NOTES
14. R. N. 

Downloaded from http://stke.sciencemag.org/ on October 26, 2015


Acknowledgments: We thank G. Urquhart for technical assistance, M. Yanagisawa and A. Suzuki for sharing 2D Etan DALT system, P. Chambron and D. Metzger for providing SMA-Cre-ER12 mice, and K. Mizuno and C. Wu for the reagents. We also thank A. Lemoff and the Protein Chemistry Core Laboratory, J. Richardson and the Molecular Pathology Core Laboratory at UT Southwestern, and the Facility for Electron Microscopy Research at McGill University for technical assistance; C. Ahn for advice on statistical analysis; B. Rothermel for discussion; and E. Olson for critical reading of the manuscript. Funding: This work was supported by grants from the NIH (RO1HL106305 to H.Y. and E.C.D.; R01HL115560 and 105314 to J.E.W.), the NIH Institutional Training in Cardiovascular Research (S03HL07380–34 to C.L.P.) and NIH Postdoctoral fellowship (F32 HL12076–1 to C.L.P.). We also thank G. Urquhart for technical assistance, M. Yanagisawa and A. Suzuki for sharing 2D Etan DALT system, P. Chambron and D. Metzger for providing SMA-Cre-ER12 mice, and K. Mizuno and C. Wu for the reagents. We also thank A. Lemoff and the Protein Chemistry Core Laboratory, J. Richardson and the Molecular Pathology Core Laboratory at UT Southwestern, and the Facility for Electron Microscopy Research at McGill University for technical assistance; C. Ahn for advice on statistical analysis; B. Rothermel for discussion; and E. Olson for critical reading of the manuscript. Funding: This work was supported by grants from the NIH (RO1HL106305 to H.Y. and E.C.D.; R01HL115560 and 105314 to J.E.W.), the NIH Institutional Training in Cardiovascular Research (S03HL07380–34 to C.L.P.) and NIH Postdoctoral fellowship (F32 HL12076–1 to C.L.P.). We also thank G. Urquhart for technical assistance, M. Yanagisawa and A. Suzuki for sharing 2D Etan DALT system, P. Chambron and D. Metzger for providing SMA-Cre-ER12 mice, and K. Mizuno and C. Wu for the reagents. We also thank A. Lemoff and the Protein Chemistry Core Laboratory, J. Richardson and the Molecular Pathology Core Laboratory at UT Southwestern, and the Facility for Electron Microscopy Research at McGill University for technical assistance; C. Ahn for advice on statistical analysis; B. Rothermel for discussion; and E. Olson for critical reading of the manuscript. Funding: This work was supported by grants from the NIH (RO1HL106305 to H.Y. and E.C.D.; R01HL115560 and 105314 to J.E.W.), the NIH Institutional Training in Cardiovascular Research (S03HL07380–34 to C.L.P.) and NIH Postdoctoral fellowship (F32 HL12076–1 to C.L.P.).
Abnormal mechanosensing and cofillin activation promote the progression of ascending aortic aneurysms in mice
Yoshito Yamashiro, Christina L. Papke, Jungsil Kim, Lea-Jeanne Ringuette, Qing-Jun Zhang, Zhi-Ping Liu, Hamid Mirzaei, Jessica E. Wagenseil, Elaine C. Davis and Hiromi Yanagisawa (October 20, 2015)
Science Signaling 8 (399), ra105. [doi: 10.1126/scisignal.aab3141]

The following resources related to this article are available online at http://stke.sciencemag.org.
This information is current as of October 26, 2015.

Article Tools
Visit the online version of this article to access the personalization and article tools:
http://stke.sciencemag.org/content/8/399/ra105

Supplemental Materials
"Supplementary Materials"
http://stke.sciencemag.org/content/suppl/2015/10/16/8.399.ra105.DC1

Related Content
The editors suggest related resources on Science's sites:
http://stke.sciencemag.org/content/sigtrans/7/330/ra57.full
http://www.sciencemag.org/content/sci/332/6027/358.full

References
This article cites 62 articles, 29 of which you can access for free at:
http://stke.sciencemag.org/content/8/399/ra105#BIBL

Permissions
Obtain information about reproducing this article:
http://www.sciencemag.org/about/permissions.dtl