Matrix mechanotransduction mediated by thrombospondin-1/integrin/YAP in the vascular remodeling

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The extracellular matrix (ECM) initiates mechanical cues that activate intracellular signaling through matrix–cell interactions. In blood vessels, additional mechanical cues derived from the pulsatile blood flow and pressure play a pivotal role in homeostasis and disease development. Currently, the nature of the cues from the ECM and their interaction with the mechanical microenvironment in large blood vessels to maintain the integrity of the vessel wall are not fully understood. Here, we identified the matricellular protein thrombospondin-1 (Thbs1) as an extracellular mediator of matrix mechanotransduction that acts via integrin αvβ1 to establish focal adhesions and promotes nuclear shuttling of Yes-associated protein (YAP) in response to high strain of cyclic stretch. Thbs1-mediated YAP activation depends on the small GTPase Rap2 and Hippo pathway and is not influenced by alteration of actin fibers. Deletion of Thbs1 in mice inhibited Thbs1/integrin αvβ1/YAP signaling, leading to maladaptive remodeling of the aorta in response to pressure overload and inhibition of neointima formation upon carotid artery ligation, exerting context-dependent effects on the vessel wall. We thus propose a mechanism of matrix mechanotransduction centered on Thbs1, connecting mechanical stimuli to YAP signaling during vascular remodeling in vivo.

extracellular matrix (ECM) | mechanotransduction | thrombospondin-1 | YAP | vessel remodeling

The extracellular matrix (ECM) is fundamental to cellular and tissue structural integrity and provides mechanical cues to initiate diverse biological functions (1). The quality and quantity of the ECM determine tissue stiffness and control gene expression, cell fate, and cell cycle progression in various cell types (2, 3). ECM–cell interactions are mediated by focal adhesions (FAs), the main hub for mechanotransduction, connecting ECM, integrins, and cytoskeleton (1, 4). In the blood vessels, an additional layer of mechanical cues derived from the pulsatile blood flow and pressure play a pivotal role in homeostasis and disease development. However, how cells sense and integrate different mechanical cues to maintain the blood vessel wall is largely unknown.

Hippo effector Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) serve as an on-off mechanosensing switch for ECM stiffness (5). YAP activity is regulated by a canonical Hippo pathway: MST1/2 (mammalian sterile 20-like 1/2 kinases) and their downstream kinases LATS1/2 (large tumor suppressor 1/2) control YAP activation via phosphorylation and retention in the cytoplasm by binding to the 14-3-3 protein (6). YAP activity is also regulated by Wnt/β-catenin and G protein-coupled receptor (GPCR) signaling pathways (7, 8). However, regulation of YAP activity is highly context dependent, and extracellular regulators are not fully understood (6, 9).

Thrombospondin-1 (Thbs1) is a homotrimeric glycoprotein with a complex multidomain structure capable of interacting with a variety of receptors such as integrins, cluster of differentiation (CD) 36, and CD47 (10). Thbs1 is highly expressed during development and reactivated in response to injury (11), exerting domain-specific and cell type–specific effects on cellular functions. We recently showed that Thbs1 is induced by mechanical stretch and is a driver for thoracic aortic aneurysm in mice (12, 13).

In the current study, we identified Thbs1 as an extracellular regulator of YAP, which is induced by mechanical stress and acts through FAs independent of actin remodeling. Mechanistically,

Significance

We propose a mechanism of matrix mechanotransduction initiated by thrombospondin-1 (Thbs1), connecting mechanical stimuli to Yes-associated protein (YAP) signaling during vascular remodeling. Specifically, Thbs1 is secreted from smooth muscle cells in response to high strain of cyclic stretch and binds to integrin αvβ1 to establish mature focal adhesions, thereby promoting the nuclear shuttling of YAP in a small GTPase Rap2- and Hippo signaling–dependent manner. We further demonstrated the biological significance of the Thbs1/integrin αvβ1/YAP pathway using two vascular injury models. Deletion of Thbs1 in mice caused maladaptive remodeling of the aorta in response to pressure overload, whereas it inhibited neointima formation upon carotid artery ligation, exerting context-dependent effects on the blood vessel wall.

The authors declare no competing interest.

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Data deposition: The secretome data have been deposited in the Japan Proteome Standard Repository/Database (jPOST), http://jpostdb.org (accession no. JPST000600/PXD013915) and RNA-seq data have been deposited in Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo/ (accession no. GSE131750). All data supporting the findings of this study are available from the corresponding authors upon reasonable request.

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Thbs1 binds integrin αβ1 and strengthens FAs, aiding in translocation of YAP to the nucleus. Deletion of Thbs1 in vivo resulted in altered vascular remodeling in response to pressure overload and flow cessation. Taken together, Thbs1 mediates dynamic interactions between mechanical stress and the YAP-mediated transcriptional cascade in the blood vessel wall.

**Results**

**Cyclic Stretch–Induced Secretion of ECM and Cell Adhesion Molecules Involved in Blood Vessel Development.** Vascular ECM is synthesized by smooth muscle cells (SMCs) from midgestation to the early postnatal period and determines the material and mechanical properties of the blood vessels (14). Owing to the long half-life of ECM, blood vessels are generally thought to stand lifelong mechanical stress without regeneration, particularly in large arteries. However, it is not completely known whether a local microremodeling system exists for the maintenance of the vessel wall. To search for a potential remodeling factor(s), we first performed cyclic stretch experiments with high strain (1 Hz; 20% strain), mimicking the pathological condition of the aortic wall. Rat SMCs were subjected to cyclic stretch for 20 h with or without brefeldin A (BFA), a protein transporter inhibitor, as a negative control for mass spectrometry analysis. Conditioned media (CM) were analyzed using quantitative mass spectrometry, and 87 secreted proteins with more than twofold differences in response to cyclic stretch were identified (Fig. 1A and B and SI Appendix, Figs. S1 and S2). Gene ontology (GO) enrichment analysis (http://geneontology.org/) revealed that proteins involved in ECM organization and structural constituents were highly enriched (Fig. 1C). Ingenuity pathway analysis (IPA) was conducted for biological interaction network among proteins in ECM–cell adhesions and blood vessel development (Fig. 1D), and Thbs1 showed multiple interactions in both categories. In addition, since we reported that Thbs1 is a mechanosensitive factor involved in aortic aneurysms (12, 13), we focused on Thbs1 for further analysis. We confirmed the increased expression and secretion of Thbs1 after cyclic stretch (Fig. 1E), suggesting that Thbs1 may play an important role in response to dynamic vessel microenvironments.

**Secreted Thrombospondin-1 Binds to Integrin αβ1 under Cyclic Stretch.** In response to cyclic stretch, SMCs showed up-regulation of the early growth response 1 (Egr1) transcription factor, phosphorylated (p) focal adhesion kinase (FAK), extracellular signal-regulated kinase (ERK), and stress-response mitogen-activated protein kinase (MAP) p38 as previously reported (SI Appendix, Fig. S3A) (13, 15, 16). Cyclic stretch altered subcellular localization of Thbs1 from the perinuclear region to the tip of the cell on the long axis (Fig. 24). We examined Thbs1 localization and reorientation of SMCs as a function of time (SI Appendix, Fig. S3 B and C). SMCs were randomly oriented after 30 min of cyclic stretch, but most cells were aligned in a perpendicular position to the stretch direction at 3 h and completely reoriented by 6 h. Thbs1 localization clearly changed from 3 h after cyclic stretch, which coincided with the reorientation of actin fibers and elongation of cells (SI Appendix, Fig. S3D). Since FAs recruit actin stress fibers (17), we costained SMCs with Thbs1 and p-paxillin, a FA molecule, under cyclic stretch. Thbs1 colocalized with p-paxillin independent of cell density after cyclic stretch (Fig. 2 B and C). Since Thbs1 has well-characterized cell surface receptors, including integrins β1, β3,
Thrombospondin-1 Regulates Maturation of the FA–Actin Complex and Controls Cell Stiffness. We next examined the effect of deletion of Thbs1 on cyclic stretch–induced formation of FA and reorientation of SMCs. Thbs1 knockout rat vascular SMCs (Thbs1KO) were generated using CRISPR-Cas9 genome editing (SI Appendix, Fig. S4). We evaluated possible off-target effects and confirmed that the expression of Cpreb3 or Tet3 was not altered in Thbs1KO cells (SI Appendix, Fig. S5 A and B). No obvious phenotypic changes were observed in Thbs1KO cells (SI Appendix, Fig. S5 C and D). Thbs1KO cells were subjected to cyclic stretch (1 Hz; 20% strain) for 8 h and were compared with control (CTRL) cells with or without 1 μM of BFA. CTRL cells responded to cyclic stretch and reoriented perpendicular to the direction of stretch, whereas BFA-treated CTRL cells and Thbs1KO cells neither elongated nor aligned to the perpendicular position (Fig. 3 A–C). Stretch-induced phosphorylation of paxillin and up-regulation of integrins αv and β1 in CTRL cells were not observed in Thbs1KO cells (Fig. 3D).

Mature FAs are localized at the termini of actin stress fibers and form a FA–actin complex (18, 19). The FA–actin complex is stabilized by vinculin deposition and integrin–talin binding, which facilitates PI3K-mediated phosphatidylinositol 3,4,5-triphosphate (PIP₃) production (20). Vinculin is present at FAs in a force-dependent manner and increases cell stiffness (21). To evaluate the maturation of FA–actin complex, we compared the vinculin expression and monitored PIP₃ levels using a GFP-tagged PIP₃ reporter (GFP-Grp1-PH) in static and stretch conditions (n = 5). Immunostaining showed that Thbs1 colocalized with integrins αv and β1 under cyclic stretch. (B) Representative immunostaining showing Thbs1 colocalization with p-paxillin under cyclic stretch (n = 5). Immunostained with p-paxillin (red), Thbs1 (green), and DAPI (blue). (C) Cartoon shows various binding domains of Thbs1 and its receptors. (E) Representative immunostaining of Thbs1 showing colocalization with integrins αv and β1 (white arrows) but not with integrin β3, CD47, or CD36 under cyclic stretch (n = 5). Immunostained for indicated antibodies (red), Thbs1 (green), and DAPI (blue). (F) Quantification of colocalization of Thbs1 with molecules shown in E using Imaris colocalization software. Bars are mean ± SEM. ***P < 0.001, two-way ANOVA. NS: not significant. (G) Immunoprecipitation (IP) with anti-Thbs1 or control immunoglobulin G (IgG) followed by Western blotting for integrins αv and β1 (white arrows) but not with integrin β3, CD47, or CD36 under cyclic stretch (n = 5). Immunostained for indicated antibodies (red), Thbs1 (green), and DAPI (blue). (H) PLA shows the clusters of Thbs1/integrin αv (red dots; white arrowheads). Bottom: highly magnified images of the white dashed box in Top. DAPI (blue) and phalloidin (green) are shown. In all experiments, rat vascular SMCs were subjected to cyclic stretch (1.0 Hz, 20% strain for 20 h in A, C, and E or 8 h in G and H). In A, C, E, and H, 80 to 100 cells were evaluated in each immunostaining. Two-way arrows indicate stretch directions. (Scale bar, 50 μm.)
conditions in CTRL and Thbs1KO cells. PIP3 was enriched on the tip of actin stress fibers which anchored FAs in stretched CTRL cells, whereas stretched Thbs1KO cells did not show recruitment of vinculin onto the FA–actin complex (Fig. 3F). To confirm this observation, we assessed mechanical properties of Thbs1KO cells using atomic force microscopy (Fig. 3G). Since cell tension positively correlates with adhesion size and its stiffness (22), we analyzed morphological changes and stiffness on the stress fibers. Stretched Thbs1KO cells showed a significant decrease in stiffness (Young’s modulus) on the stress fibers compared with stretched CTRL cells, although the height of stretched Thbs1KO cells was comparable to that of stretched CTRL cells (Fig. 3H). Taken together, our findings suggest that Thbs1 is required for maturation of the FA–actin complex and maintenance of cellular tension, thereby correctly orienting actin fibers in response to cyclic stretch.

**Thrombospondin-1 Regulates Nuclear Shuttling of YAP via Integrin αβ1 in a Rap2-Dependent Manner.** To understand the molecular basis of Thbs1 contribution in mechanotransduction, we performed RNA sequencing (RNA-seq) using CTRL and Thbs1KO cells in static and cyclic stretch conditions (SI Appendix, Fig. S6). In the static condition, RNA-seq revealed 38 and 47 genes that were differentially regulated in CTRL and Thbs1KO cells, respectively. Conversely, 126 genes in CTRL cells and 163 genes in Thbs1KO cells were differentially regulated in CTRL and Thbs1KO cells, re- spectively. With the exception of gene encoding mesothelin (Msln), the expressions of YAP target genes were markedly down-regulated in Thbs1KO cells, suggesting that Thbs1 strongly controls YAP target genes in the stretch condition (SI Appendix, Fig. S6) and may be a critical component of the YAP signaling pathway.

Next, to elucidate the role of Thbs1 in the mechanical regulation of YAP, we examined YAP localization with or without Thbs1 under cyclic stretch. Consistent with a previous report

**Fig. 3.** Deletion of Thbs1 affects maturation of focal adhesion and cell stiffness. (A) Wild-type cells with or without 1 μM of BFA or Thbs1KO cells were subjected to cyclic stretch (1.0 Hz, 20% strain, 8 h). Two-way arrows indicate the stretch direction. (Scale bars, 50 μm.) Phalloidin (red) is also shown. (B and C) The orientation of each cell was analyzed by measuring the orientation angle (θ) of the ellipse relative to the stretch axis (n = 3). (D) Thbs1KO cells show reduced FA formation. Immunostaining with p-paxillin (red), integrin αv (red), integrin β1 (red), Thbs1 (green), and DAPI (blue) (n = 3). Phalloidin (red) is also shown. White arrows indicate colocalization with Thbs1. Two-way arrows indicate stretch direction. (Scale bars, 50 μm.) (E) Representative immunostaining of CTRL or Thbs1KO cells with vinculin (red) in static or stretch condition. GFP-GRP1-PH (green) and DAPI (blue) are also shown. White arrows show vinculin deposition onto the tip of actin fibers (n = 3). Two-way arrows indicate stretch direction. (Scale bars, 50 μm.) (F) Immunostaining of CTRL or Thbs1KO cells with vinculin (green) and phalloidin (red). Rat vascular SMCs were subjected to cyclic stretch (1.0 Hz, 20% strain for 8 h). Two-way arrows indicate stretch direction. (Scale bars, 25 μm.) Arrow heads (purple) show vinculin deposition onto the tip of actin fibers. (G) Young’s modulus of actin fibers in CTRL (n = 129) or Thbs1KO (n = 86) cells measured using atomic force microscopy. Representative topographic images and stiffness maps are shown. (H) Cell height and Young’s modulus are shown. Bars are means ± SEM. ***P < 0.001, unpaired t test. NS: not significant.
(23), cyclic stretch induced nuclear shuttling of YAP in CTRL cells (Fig. 4A). Although YAP was retained in the cytoplasm after cyclic stretch in Thbs1KO cells (Fig. 4A), exogenously added recombinant human THBS1 (rhTHBS1) promoted nuclear shuttling of YAP after cyclic stretch (Fig. 4B and SI Appendix, Fig. S8). Since nuclear shuttling of YAP is regulated by a kinase cascade of the Hippo pathway, we examined p-YAP and p-LATS levels using Western blotting. In CTRL cells, p-YAP and p-LATS were decreased in stretch condition, whereas p-YAP and p-LATS persisted in Thbs1KO cells (Fig. 4C). These observations indicate that Thbs1 controls nuclear shuttling of YAP in a Hippo pathway–dependent manner. The Ras-related GTPase Rap2 was recently identified as a negative regulator of the Hippo pathway and as an inhibitor of YAP activation induced by ECM stiffness (30). To determine whether stretch- and Thbs1-induced nuclear shuttling of YAP is regulated by Rap2 activity, we measured active Rap2 (Rap2-GTP) levels in CTRL or Thbs1KO cells in static and stretch conditions using the Ral-GDS-RBD pull-down assays. As shown in Fig. 4D, active Rap2 decreased in CTRL cells after stretch but not in Thbs1KO cells. To further determine the relationship between Rap2 activity and nuclear shuttling of YAP, CTRL or Thbs1KO cells were transfected with Myc-Rap2A (wild type [WT]), Myc-Rap2A (G12V) constitutively active mutant, or Myc-Rap2A (S17N) (31). Rap2-GTP was strongly retained in the cytoplasm of Thbs1KO cells but not in CTRL cells, which indicates that Rap2 activity is regulated by Thbs1. Thus, Thbs1 controls nuclear shuttling of YAP via integrin αvβ1 in a Rap2-dependent manner. (A) Representative immunostaining of CTRL or Thbs1KO cells in the static or stretch condition with YAP (green), Phalloidin (red) and DAPI (blue) are also shown. Two-way arrows indicate stretch direction. (Scale bars, 50 μm.) Quantification of YAP localization is shown on the Right. In each experiment 150 to 200 cells were evaluated (∗∗∗P < 0.001, one-way ANOVA). (B) Western blot shows Thbs1, p-YAP, and p-LATS levels in CTRL and Thbs1KO cells in the static or stretch condition (∗∗∗P < 0.001). Quantification graphs are shown on the Right. Bars are means ± SEM. (C) Pull down of GTP-bound Rap2 from CTRL and Thbs1KO cells in static and stretch conditions using Ral-GDS-RBD agarose beads (∗∗P < 0.01, one-way ANOVA). (D) Overexpression of Myc-Rap2A (WT), constitutively active Myc-Rap2A (G12V), or dominant-negative Myc-Rap2A (S17N) in CTRL and Thbs1KO cells in the static or stretch condition (∗∗∗P < 0.001). Representative immunostaining with Myc (red), YAP (green), and DAPI (blue). Two-way arrows indicate stretch direction. (Scale bars, 50 μm.) Quantification of YAP localization is shown on the Right. In each experiment 30 to 100 cells were evaluated (∗∗∗P < 0.001). Quantification of YAP localization is shown on the Right. In each experiment 150 to 200 cells were evaluated (∗∗∗P < 0.001). qPCR data for confirming the knockdown of Itgav, Itgb1, and Itgb3 are provided in SI Appendix, Fig. S10.
dominant negative mutant. Forty-eight hours after transfection, cells were subjected to cyclic stretch (1.0 Hz, 20% strain) for 8 h and immunostained with antibodies directed against Myc and YAP and stained with DAPI to identify nuclei. Rap2A activation (G12V) cancelled nuclear shuttling of YAP under cyclic stretch in CTRL cells. In contrast, Rap2A inactivation (S17N) forced YAP nuclear shuttling with or without Thbs1, overriding the regulation of Thbs1. These data suggested that Thbs1 controls nuclear shuttling of YAP in a Rap2A-dependent manner.

Tension on actin fibers is also important for regulation of YAP, dependent on or independent of the formation of FAs (31–33). We assessed how actin dynamics and inhibition of FAK affects stretch-induced nuclear shuttling of YAP. CTRL cells were subjected to cyclic stretch for 8 h with the actin polymerization inhibitor cytochalasin D (CytoD) or actin depolymerization inhibitor jasplakinolide (Jasp) or FAK inhibitor PF-00562271 (FAKi). The efficiency of the treatment was evaluated by measuring the orientation angle (θ) of the long axis of the ellipse relative to the cyclic stretch direction (SI Appendix, Fig. S9 A and B). Although CytoD and Jasp showed no effects on nuclear shuttling of YAP under cyclic stretch, YAP remained in the cytoplasm in FAKi-treated cells (SI Appendix, Fig. S9 C and E). Furthermore, although overexpression of slingshot1 (Ssh1, Shb1OH) or inactivation of Shb1, a phosphatase of cofilin, induced depolymerization of actin fibers through dephosphorylation of cofilin, it did not affect YAP localization under cyclic stretch (SI Appendix, Fig. S9 D and E). These observations suggest that stretch-induced nuclear shuttling of YAP is dependent on FAs but not on actin polymerization. To confirm this, we performed small interfering RNA (siRNA)-mediated knockdown of integrins αv (siILt7γv), β1 (siILt7β1), and β3 (siILt7β3) along with scramble siRNA (SI Appendix, Fig. S10) followed by cyclic stretch. As expected, knockdown of integrin αv or β1 was sufficient to inhibit stretch-induced nuclear shuttling of YAP (Fig. 4F). We next investigated whether matrix proteins in general serve as an extracellular regulator of YAP under cyclic stretch. Since fibronectin has been shown to regulate the Hippo pathway through FAK-src-P13K during cell spreading (34) and is a ligand for integrin αvβ1, we knocked down fibronectin in SMCs and subjected them to cyclic stretch to assess its impact on YAP localization. As SI Appendix, Fig. S11 shows, fibronectin knockdown had no effect on nuclear shuttling of YAP in response to stretch. We also assessed whether Thbs1-mediated nuclear shuttling of YAP is observed in endothelial cells. Using human umbilical vein endothelial cells (HUVECs), we found that cyclic stretch did not induce phosphorylation of paxillin or localization of Thbs1 to FAs, even though Thbs1 was secreted into CM (SI Appendix, Fig. S12 A and B). Actin fibers aligned in a perpendicular position, and contact inhibition was reduced by cyclic stretch, but YAP remained in the cytoplasm of HUVECs (SI Appendix, Fig. S12 C and D), suggesting that regulation of YAP by Thbs1 under cyclic stretch condition is specific to vascular SMCs. Taken together, these data indicate that stretch-induced, Thbs1-mediated nuclear shuttling of YAP is dependent on integrin αvβ1 and that effect is specific to SMCs.

Thrombospondin-1/Integrin/YAP Signaling Is Involved in Mechanical Stress–Induced Vascular Pathology. An in vivo model was used to evaluate whether Thbs1-mediated YAP regulation is involved in the pathogenesis of vascular diseases. We previously reported that transverse aortic constriction (TAC) induced Thbs1 expression (12). Concordantly, Thbs1 was induced in endothelial cells (ECs), SMCs, and adventitial cells after TAC was performed in WT mice and PLA showed the interaction between Thbs1 and integrin β1 in these cells (Fig. 5A). We next evaluated the effect of loss of Thbs1/integrin β1 in TAC-induced aortic remodeling using Thbs1 null (Thbs1KO) mice. Whereas 100% of WT mice survived at 5 wk after TAC (n = 13), 31.9% (7/22) of Thbs1KO mice died, with three showing aortic rupture (Fig. S5 B and SI Appendix, Fig. S13A). The heart weight (HW) and body weight (BW) ratio (HW/BW) was significantly higher in TAC-operated Thbs1KO mice than in WT mice, in accordance with a previous report (35) (Fig. 5C). In addition, TAC-operated Thbs1KO aortas had enlarged aortic lumen (Fig. 5D), and dissected aortas showed severely disrupted elastic fibers (Fig. 5E). Morphometric analysis showed a significant increase in the internal elastic lamella (IEL) perimeter, outer perimeter, and total vessel area in TAC-operated Thbs1KO aortas compared to TAC-operated WT aortas (Fig. 5F).

Finaly, we employed carotid artery ligation injury to address the contribution of Thbs1/integrin/YAP signaling in flow cessation–induced neointima formation (Fig. 6D). Thbs1 has been shown to be required for neointima formation (36), and YAP mediates phenotypic modulation of SMCs during neointima formation (37). Time course analyses of the ligated arteries revealed that Thbs1 was up-regulated in the inner layer at 1 wk after ligation when YAP expression was not evident (Fig. 6B and SI Appendix, Fig. S14). At 2 wk after ligation, nuclear YAP peaked in the developing neointima (Fig. 6C) and coexpressed with Thbs1 (Fig. 6B and SI Appendix, Fig. S15). At 3 wk, Thbs1 and YAP expression decreased as the neointima became stabilized (Fig. 6B and SI Appendix, Fig. S16). YAP targets CTGF, Serpine1, CYR61, and Caveolin-3 were highly expressed in the neointima (SI Appendix, Fig. S17). PLA showed the interaction between Thbs1 and integrin β1 in the neointima (left carotid artery [LCA] in Fig. 6D) but not in the contralateral artery (right carotid artery [RCA] in Fig. 6D). To confirm the relationship between Thbs1/integrin/YAP and neointima formation, we injected the lentivirus (LV)-encoding Thbs1 siRNA (siThbs1) into WT mice through tail vein injection at 2 wk after ligation and evaluated neointima at 3 wk. GFP-LV injection from the tail vein showed clear expression of GFP in the neointima, endothelial cells of the right carotid artery, and the liver (SI Appendix, Fig. S18), confirming the delivery of siRNA into the neointima. As we expected, the neointima was not observed in siThbs1 virus-treated animals, similar to Thbs1KO mice, and YAP levels and nuclear localization were markedly decreased as Thbs1 expression decreased (Fig. 6 C and E).

Discussion
In this study, we identified Thbs1 as a critical molecule involved in mechanotransduction and extracellular regulation of YAP, leading to dynamic remodeling of the blood vessels. We found that Thbs1, once secreted in response to mechanical stress, binds to integrin αvβ1 and aids in the maturation of the FA–actin complex, downregulating Rap2 activity, thereby mediating activation of YAP. Loss of Thbs1 decreases cell stiffness in vitro, and deletion or down-regulation of Thbs1 in mice alters cellular behavior in response to mechanical stress in a context-dependent manner. Taken together, we propose that the Thbs1/integrin/YAP signaling pathway plays a critical role in vascular remodeling in vivo (Fig. 7).
Fig. 5. Thbs1 deficiency in mice results in aortic dissection under pressure overload. (A) Immunostaining of Thbs1 (red) in sham or TAC-operated aortas in WT mice (Top). PLA showing the interaction between Thbs1 and integrin β1 (red dots in Bottom). Right shows highly magnified images of white dashed boxes in Bottom. Arrowheads (white) show PLA cluster. DAPI (blue) and autofluorescence of elastin (green) are also shown. n = 3. (Scale bars, 50 μm.) (B) Kaplan-Meier survival curve for WT (n = 13) and Thbs1KO (n = 22) mice after TAC. *P = 0.043, log-rank test. (C) HW/BW in sham or TAC-operated mice. Bars are mean ± SEM. *P < 0.05, ***P < 0.001, quantification by the Kruskal–Wallis test with Dunn multiple comparisons is shown. The number of animals is indicated in each bar. (D) Hematoxylin and eosin staining of the ascending aortas for histological comparison between sham and TAC-operated aortas. (Scale bars, 100 μm.) (E) Aortic dissection was observed in Thbs1KO ascending aorta proximal to the constriction. (Scale bars, 100 μm.) (F) Morphometric analysis showing the IEL perimeter, outer perimeter, total vessel area, and wall thickness. Bars are mean ± SEM. *P < 0.05, ***P < 0.001, one-way ANOVA. NS: not significant. The number of animals is indicated in each bar. (G) Immunostaining of TAC-operated aortas with YAP (red) in WT and Thbs1KO mice. On the Right, highly magnified images of the white dashed boxes in the adventitial (Adv.) and medial (Med.) layers in the Left are shown. DAPI (blue) and autofluorescence of elastin (green) are also shown. n = 3. (Scale bars, 50 μm.) (H) Representative images of immunohistochemistry for CTGF in TAC-operated aortas of WT and Thbs1KO mice (n = 3 per genotype). The outer elastic lamella as a border between the medial layer and adventitia is shown by the green dashed line. Arrowheads (red) indicate CTGF-positive cells in the medial layer. (Scale bars, 100 μm.)
Vinculin at the tip of actin fibers. Currently, the precise ciliated maturation of FA complex by inducing deposition of bound to integrin is involved in cell adhesions, elastic fiber formation, and TGF-β cyclic stretch induced secretion of various ECM proteins in mass spectrometry and GO analysis, we found that high strain of transduction cascade are not completely understood. Using modeling of the vessel wall, and how it is connected to the mechanical stretch and secretory pathway are interconnected molecules involved in these biological processes. Interestingly, the proteolytic remodeling of molecules, indicating that Thbs1 may act as a feedforward driver for a mutant cell line lacking YAP showed decreased Thbs1 levels by controlling FA assembly (41). A previous study described that Rap2 negatively regulates nuclear shuttling of YAP in a Hippo pathway–dependent manner. These authors proposed a cascade of signaling triggered by FAs → PLCγ1 (phospholipase C gamma 1) → PtdIns(4,5)P2 (phosphatidylinositol 4, 5-bisphosphate) → PA (phosphatidic acid) → PDZGEF (RAPGEF) → Rap2 → MAP4K → LATS → YAP (30) and showed that increased matrix stiffness led to inactivation of Rap2 and nuclear shuttling of YAP. It was also proposed that this signaling axis works in parallel with RhoA, which mediates cytoskeletal tension during cell spreading (5, 32). Our data showed that stretch-induced YAP activation is Rap2 dependent and is not influenced by altered actin fibers. Therefore, it is possible that Thbs1 mediates nuclear shuttling of YAP by inactivating Rap2 through altering the composition of FA or by controlling FA assembly (41). A previous study described that a mutant cell line lacking YAP showed decreased Thbs1 levels along with down-regulation of YAP target genes and FA molecules, indicating that Thbs1 may act as a feedforward driver for YAP signaling (41). Interestingly, the proteolytic remodeling of laminin-1 has been shown to modulate awakening of dormant cancer cells through integrin/FAK/ERK/MLCK (myosin light chain kinase)/YAP signaling (42), and fibroblast-derived periostrin (encoded by Postn) has been shown to mediate colorectal

**Mechanical Stress and Micro- and Macroremodeling of the Vessel Wall by ECM.** Vascular ECM, a major component of the vessel wall, is subjected to repeated mechanical stress throughout life. Due to its long half-life [for example, 50 to 70 y for elastic fibers of the human artery (38)], the vascular ECM is considered to be difficult to regenerate once it is damaged by physical and/or biochemical insults. Although ECM synthesis via mechanical stretch was previously described (39, 40), its biological significance, whether it contributes to microremodeling or dynamic remodeling of the vessel wall, and how it is connected to the mechanotransduction cascade are not completely understood. Using mass spectrometry and GO analysis, we found that high strain of cyclic stretch induced secretion of various ECM proteins involved in cell adhesions, elastic fiber formation, and TGF-β signaling. Using IPA, Thbs1 was identified as one of the key molecules involved in these biological processes. Interestingly, mechanical stress and secretary pathway are interconnected since BFA treatment or deletion of Thbs1 led to the alteration of the mechanoresponse in SMCs and disrupted alignment of cells in response to cyclic stretch. Our observations indicate that mechanical stress induces secretion of Thbs1, which initiates mechanotransduction and activates the integrin/YAP pathway, thereby remodeling the vessel wall to accommodate the changes in mechanical microenvironment. Thbs1 and integrins are essential components of matrix mechanotransduction, serving as biomechanical ligand and sensors of the microenvironment.

**Thbs1 as an Extracellular Mediator of YAP Signaling Pathway.** We showed that mechanical stretch–induced, secreted Thbs1 directly bound to integrin αvβ1 and colocalized with p-paxillin, and facilitated maturation of FA complex by inducing deposition of vinculin at the tip of actin fibers. Currently, the precise mechanism by which secreted Thbs1 is recruited to integrin αvβ1 is unclear. However, we speculate that Thbs1 ligation to integrins activates downstream molecules, leading to maturation of FAs and strengthening of actin fibers. Interestingly, a loss of Thbs1 decreased cell stiffness and altered YAP target gene expression after cyclic stretch. We further showed that stretch-downregulated Rap2 activity in CTRL cells, whereas Rap2 activity remained high in the absence of Thbs1, which forced YAP retention in the cytoplasm. Our observation agrees with the recent study by Meng et al. demonstrating that Rap2 negatively regulates nuclear shuttling of YAP in a Hippo pathway–dependent manner. These authors proposed a cascade of signaling triggered by FAs → PLCγ1 (phospholipase C gamma 1) → PtdIns(4,5)P2 (phosphatidylinositol 4, 5-bisphosphate) → PA (phosphatidic acid) → PDZGEF (RAPGEF) → Rap2 → MAP4K → LATS → YAP (30) and showed that increased matrix stiffness led to inactivation of Rap2 and nuclear shuttling of YAP. It was also proposed that this signaling axis works in parallel with RhoA, which mediates cytoskeletal tension during cell spreading (5, 32). Our data showed that stretch-induced YAP activation is Rap2 dependent and is not influenced by altered actin fibers. Therefore, it is possible that Thbs1 mediates nuclear shuttling of YAP by inactivating Rap2 through altering the composition of FA or by controlling FA assembly (41). A previous study described that a mutant cell line lacking YAP showed decreased Thbs1 levels along with down-regulation of YAP target genes and FA molecules, indicating that Thbs1 may act as a feedforward driver for YAP signaling (41). Interestingly, the proteolytic remodeling of laminin-1 has been shown to modulate awakening of dormant cancer cells through integrin/FAK/ERK/MLCK (myosin light chain kinase)/YAP signaling (42), and fibroblast-derived periostrin (encoded by Postn) has been shown to mediate colorectal.

**Fig. 6.** Thbs1 regulates YAP expression during neointima formation upon carotid artery ligation. (A) Diagram of LCA ligation. (B) Immunostaining of cross sections of ligated arteries with YAP (red) and Thbs1 (white) at 1 wk (n = 5), 2 wk (n = 5), and 3 wk (n = 4) after ligation in WT mice. DAPI (blue) and autofluorescence of elastin (green) are also shown. Quantification of colocalization with YAP and DAPI using Imaris colocalization software is indicated in yellow (number of nuclear YAP/total number of nuclei). (Scale bars, 50 μm.) (C) Quantification of colocalization between YAP and DAPI in B and E using Imaris colocalization software. Bars are mean ± SEM. **P < 0.01, ***P < 0.001, one-way ANOVA. (D) PLA showing cluster of Thbs1/integrin β1 (red dots, yellow arrowheads) at 3 wk after ligation in RCA and LCA. DAPI (blue) and autofluorescence of elastin (green) are also shown. (Scale bars, 50 μm.) n = 3. (E) Cross sections of ligated arteries at 3 wk postinjury in WT (n = 4), siThbs1-treated WT mice (n = 4), and Thbs1KO (n = 5) mice. Immunostaining with YAP (red), Thbs1 (white), DAPI (blue), and autofluorescence of elastin (green). Note neointima formation in WT arteries. Yellow arrowheads show nuclear localization of YAP. (Scale bars, 50 μm.)
tumorigenesis through integrin/FAK/Src/YAP signaling (43), further supporting the idea that an ECM can regulate YAP signaling in various biological conditions.

Role of Matrix Mechanotransduction in Vessel Wall Remodeling. Recent studies have shown that ECM mechanical cues mediate cell proliferation, differentiation, autophagy, and lipid metabolisms by regulating focal adhesions (1, 44–47). Thbs1 is present at low levels in the postnatal vessels and up-regulated in various vascular diseases such as atherosclerosis (48), ischemia reperfusion injury (49), aortic aneurysm (13), and pulmonary arterial hypertension (50). YAP is also known to play a role in an emergency stress response to maintain tissue homeostasis (51, 52). As we describe here, Thbs1KO mice promoted maladaptive remodeling in response to pressure overload and showed higher mortality after TAC, increased occurrence of aortic dissection, and lack of up-regulation of YAP in medial SMCs. In WT mice, YAP was induced and activated in adventitial cells and SMCs, leading to proliferation and hypertrophy, respectively, and maintenance of wall tension during aortic wall dilatation. Since Ctgf null mice exhibit higher mortality after TAC (53), CTGF may be one of the YAP target genes crucial for response to pressure overload. Although Thbs1-dependent YAP nuclear shuttling in the medial SMCs was not as clear as that of in vitro assays, our data suggest that the formation of FAs by Thbs1 and induction of Thbs1/integrin/YAP signaling play a protective role in response to pressure overload, presumably by increasing cell stiffness. Suppression of Thbs1/integrin/YAP signaling reduced proliferation of neointimal cells upon carotid artery ligation and thus had a beneficial effect on flow cessation–induced maladaptive vascular remodeling. The reasons for this discrepancy between the TAC model and flow cessation model may be that different mechanical stresses induce a distinct subset of YAP target genes and lead to vascular remodeling, although a full characterization of Thbs1-dependent YAP targets in different injury models is warranted. Taken together, our observations emphasize the highly context dependent action of Thbs1/integrins/YAP in remodeling of the vessel wall.

Materials and Methods

Detailed descriptions are provided in SI Appendix.

Cell Culture and Reagents. Rat vascular SMCs (Lonza, R-ASM-580, isolated from the aorta of 150 to 200 g adult male Sprague-Dawley rats) were grown in Dulbecco’s modified Eagle medium (DMEM) with 20% (vol/vol) fetal bovine serum (FBS) and 1 × Antibiotic-Antimycotic (ThermoFisher Scientific). HUVECs were grown in HuMedia-MvG (KURABO) supplemented with 5% (vol/vol) FBS and growth factors. The cell lines were used between passages 7 and 12 and tested for mycoplasma contamination using a mycoplasma detection set (TaKaRa, #6601) following the manufacturer’s protocol (the result is provided in SI Appendix, Fig. S19). Brefeldin A (B5936) was purchased from Sigma-Aldrich. FAK inhibitor PF-00562271 (S2672) was purchased from Selleck. Cytochalasin D (037-17561) and latrunculin A (125-04363) were purchased from Wako. Jasplakinolide (AG-CN2-0037-C050) was purchased from AdipoGen Life Sciences. Recombinant human thrombospondin-1 was purchased from R&D Systems (3074-TH-050). Mice. C57BL/6J (wild-type) mice were purchased from Charles River, and Thbs1 null mice were purchased from Jackson Laboratory (B6.129S2-Thbs1tm1Hyn/J) and crossed with C57BL/6 mice for line maintenance. Male mice were used for experiments to exclude hormonal regulation by estrogen. All mice were kept on a 12 h/12 h light/dark cycle under specific pathogen-free conditions, and all animal protocols were approved by the Institutional Animal Experiment Committee of the University of Tsukuba.
**Statistical Analysis.** All experiments are presented as means ± SEM. Statistical analysis was performed using Prism 7 (Graph Pad, version 7.0e). A Shapiro-Wilk test was used for the normality test. Statistical significance was determined by either unpaired t test, one-way ANOVA, or two-way ANOVA followed by Tukey’s multiple comparison test. If the normality assumption was violated, nonparametric tests were conducted. A Kruskal-Wallis test was used with Dunnett’s multiple comparisons was used in Fig. S5. To estimate the cumulative survivals, Kaplan–Meier survival curves and a long-rank test were used in Fig. S5. P < 0.05 denotes statistical significance.

**Data Availability.** The raw data were deposited in the Japan Proteome Standard RepositoryDatabase (iPost; https://iPostdb.org; the accession number is JPTST006000/PXD103915). The RNA-seq data were deposited in National Center for Biotechnology Information’s (NCBI)'s Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/); the GEO accession number is GSE131750 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE131750).

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